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Fungal Decomposition of Leaf Litter in a Cool Temperate Forest

Takashi Osono

2002

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Contents

General Introduction

Forest ecosystems consist of the interaction of two subsystems, belowground decomposition subsystem and aboveground plant subsystem (Takeda 1994). The function of the decomposition subsystems includes the recycling of essential nutrients available to plant roots and the formation of soil organic matters that retain the nutrients (Swift et al. 1979). Litter decomposition in the decomposition subsystem has been studied for the understanding of the mechanism of maintenance of functioning and biodiversity in forest ecosystems (Takeda 1994).

Chemical aspects of litter decomposition processes have been studied intensively. The rates of litter decomposition is reviewed in Takeda et al. (1987) and Takeda (1998). The dynamics of nitrogen, one of essential elements that limit not only plant growth but also growth of decomposer microbes, is well described (Berg 1986; Takeda et al. unpublished; Osono and Takeda unpublished). Leaching, immobilization and mobilization of nitrogen in decomposing litter have been related to the availability of organic chemical energy sources to decomposers (Melillo et al. 1989; Aber et al. 1990; Osono and Takeda unpublished). In temperate forests, the availability of organic molecules to decomposers decreased as freshly fallen litter transformed to soil organic matter due to selective degradation of holocellulose (Aber et al. 1990). On the other hand, a few studies have been carried out to relate the ecology and functioning of decomposer organisms to organic chemical and nutrient dynamics during litter decomposition.

Fungi play an important role in litter decomposition than other decomposer organisms. This is because: (i) fungi contribute about 70% of total respiration of soil organisms (Anderson and Domsch 1975; Parkinson et al. 1978; Kjøller and Struwe 1982; Bewley and Parkinson 1985; Alphei et al. 1995; Zhang and Zak 1998); (ii) fungi can attack the lignocellulose matrix in litter which other organisms are unable to assimilate (Frankland et al. 1982; Cooke and Rayner 1984); and (iii) fungi consist of filamentous cells called hyphae

that can easily penetrate into three dimensional litter structure. Fungal populations cause the chemical changes of litter by the production of extracellular enzymes, but there has been no study that relate the ecology and functioning of fungal community to the organic chemical and nutrient dynamics during litter decomposition.

Ecological studies of litter decomposing fungi have been focused on successional patterns of occurrence during decomposition (Hudson 1968). Some authors examined the functional role of fungi in litter decomposition (Kjøller and Struwe 1980, 1987, 1992; Sinsabaugh et al. 1991; Sinsabaugh 1994) and demonstrated the importance of the Basidiomycota in lignocellulose decomposition in litter (Lindeberg 1946; Saito 1957, 1965; Hintikka 1970). However, in nature, leaf litter usually follows a different decay pattern from those litter colonized by the Basidiomycota, because their mycelia are often unevenly distributed, especially because of their tendency to develop as fairy-rings and to form a mosaic of individuals in forest floors (Hintikka 1970, 1982; Swift 1982). Furthermore, litter is colonized by a wide variety of fungi, including not only the Basidiomycota but also the Ascomycota and the Zygomycota (Hudson 1968). Therefore, functional biodiversity of fungi should be examined to evaluate the functioning of fungal community in litter decomposition.

The purpose of this study is to clarify the mechanism of fungal decomposition of leaf litter of Japanese beech. The dynamics of organic matter and nutrients was related to the successive colonization of fungal populations in a litter decomposition experiment in the field. Then, the functional roles of fungi populations were verified with pure culture decomposition tests.

In Chapter 1, methodology of fungal isolation from beech leaf litter was developed. In the following two chapters, mycological survey was carried out for species composition of microfungi (mostly the Zygomycota and the Ascomycota) on individual leaf (Chapter 2) and for that of macrofungi (the Basidiomycota) on forest floor (Chapter 3). In Chapter 4, fungal ingrowth and succession during litter decomposition was directly related to changes in organic chemical constituents and nutrients over a 3 year period using the litter-bag method.

The relationship between fungal colonization and decay phases of litter was evaluated. In Chapter 5, functional biodiversity of fungi on beech litter were investigated and their litter decomposition potentialities were verified with pure culture decomposition test with special reference to their ability to decomposer lignocellulose. In Chapter 6, functional species responsible for litter decomposition were inoculated to not only freshly fallen litter but also partly decomposed litter that were different in substrate quality to assess the ability to attack residual recalcitrant substances in partly decomposed litter. In General Discussion, these results were summarized and the mechanism of fungal decomposition of beech leaf litter and its implication for soil humus accumulation were discussed.

Material and study site

Japanese beech (*Fagus crenata* Bl.) was chosen as the material because: (i) beech is a dominant tree species in cool temperate regions in Japan and *Fagus* forests are major components of terrestrial ecosystems on the earth (Hara 1996); (ii) beech leaves follows decomposition pattern typical of temperate tree species and its slowly decomposing leaves are suitable for a long term study (Anderson 1973; Gosz et al. 1973; Melillo et al. 1982; Takeda et al. 1987; Wise and Schaefer 1994; Rutigliano et al. 1998; Zeller et al. 2000, 2001); and (iii) a variety of fungal taxa occur as decomposers (Saito 1956; Hogg and Hudson 1968; Tokumasu and Tubaki 1982; Kloidt et al. 1987; Kjøller and Struwe 1990; Chasseur and Beguin 1990; Chasseur 1992; Osono and Takeda 1999b, 2001b).

A cool temperate deciduous forest was chosen as the study site in the Ashiu Experimental Forest of Kyoto University (35°18'N, 135°43'E) at Miyama town, Kita-kuwata county, Kyoto prefecture, Japan. Mean annual temperature is about 10°C and mean monthly temperature ranged from 0.4°C in January to 25.5°C in August. The mean annual precipitation over a 56 year period is 2495 mm. The study area was covered with snow during the winter period from December to April.

Beech is distributed along forest slopes in the site (Group for the study on ecology of natural forest 1972). Upper and lower parts of slopes show moder and mull soils, respectively (Takeda and Kaneko 1988). The upper and lower sites were used in fruit body observation of the Basidiomycota (Chapter 3) and decomposition experiment of leaf litter (Chapter 4). It has been commonly recognized that the litter decomposition rate is faster in mull soil than in moder and mor soils (Bocock et al. 1960; Bocock 1964; Swift et al. 1979; Takeda et al. 1987).

The soil property of the upper and lower sites is shown in Table I.2. The vegetation of the study site is shown in Table I.3. The vegetation of the upper site is mostly characterized by *F. crenata*, *Clethra barvinervis*, *Ilex macropoda*, *Magnolia salicifolia*, and *Lyonia ovalifolia*, while that of the lower site is mostly characterized by *F. crenata*, *Q. mongolica* var.

grosseserrata, *Acer mono* var. *marmoratum*, *Carpinus laxiflora*, and *Acer palmatum* subsp. *amoenum*.

In this site, decomposition of leaf litter have already been studied for various tree species in terms of decomposition rate and organic chemical and nutrient dynamics (Takeda et al. 1987; Takeda et al. unpublished; Osono and Takeda unpublished).

Table I.2 Soil characteristics of the upper and lower sites.
(after Tateno et al., unpublished).

	Upper	Lower
pH (KCl)	3.1	3.6
pH (H_2O)	4.1	4.4
Carbon %	6.5	9.1
Nitrogen %	0.35	0.68
C/N	18.5	13.4
Calcium (ppm)	2.9	34.6
Magnesium (ppm)	5.3	9.1
Potassium (ppm)	2.5	6.0
Water content %	49.1	71.9
Bulk density (g dry soil cm^{-3})	0.60	0.30
Pool size of inorganic nitrogen	0.74	1.02
Net mineralization	2.45	7.29
Nitrification	0.01	2.85
% nitrification	0.3	47.9

Table I.3 Species composition of tree species (diameter at breast height >5cm) in the study site (after Tateno et al., unpublished). BA, basal area (cm^2).

Tree species	Number	Number%	BA	BA%
Fagus crenata Blume	35	9.6	20109	27.23
Quercus mongolica var. *grosseserrata* (Blume) Rehder et Wilson	7	1.9	11508	15.59
Acer mono subsp. *marmoratum* (Nichol.) Kitamura	8	2.2	6390	8.65
Acer sieboldianum Miq.	26	7.1	4735	6.41
Clethra barvinervis Sieb. et Zucc.	100	27.4	4663	6.32
Carpinus laxiflora (Sieb. et Zucc.) Blume	11	3.0	4040	5.47
Carpinus tschonoskii Maxim.	7	1.9	3828	5.18
Lyonia ovalifolia subsp. *neziki* Hara	23	6.3	2521	3.41
Sorbus alnifolia (Sieb. et Zucc.)	10	2.7	2316	3.14
Acanthopanax sciadophylloides Fr. et Sav.	8	2.2	1804	2.44
Cornus controversa Hemsl.	2	0.5	1374	1.86
Fraxinus sieboldiana Blume	10	2.7	1364	1.85
Ilex macropoda Miq.	12	3.3	1204	1.63
Acer nipponicum Hara	12	3.3	1171	1.59
Pieris japonica (Thunb.) D. Don	25	6.8	1005	1.36
Magnolia salicifolia (Sieb. et Zucc.)	16	4.4	785	1.06
Sorbus americana subsp. *japonica* (Maxim.) Kitamura	7	1.9	734	0.99
Lindera umbellata Thunb.	1	0.3	607	0.82
Pterostyrax hispida Sieb. et Zucc.	3	0.8	562	0.76
Hamamelis japonica subsp. *obutusata* (Matsumura) Sugimoto	6	1.6	439	0.59
Evodiopanax innovans (Sieb. et Zucc.) Nakai	3	0.8	438	0.59
Acer palmatum subsp. *amoenum* (Carr.) Hara	5	1.4	412	0.56
Styrax obassia Sieb. et Zucc.	2	0.5	384	0.52
Aseculus turbinata Blume	1	0.3	373	0.51
Ilex leucoclada (Maxim.) Makino	5	1.4	260	0.35
Acer japonicum Thunb.	4	1.1	161	0.22
Wisteria floribunda (Wild)DC.	2	0.5	153	0.21
Cornus kousa Buerger ex Hance	6	1.6	146	0.20
Malus tschonoskii (Maxim.) C. K. Schneider	1	0.3	129	0.17
Rhus trichocarpa Miq.	2	0.5	91	0.12
Viburnum plicatum f. *tomentosum* (Thunb.) Rehder	2	0.5	46	0.06
Prunus incisa subsp. *kinkiensis* (Koudz.) Kitamura	1	0.3	31	0.04
Schizophragma hydrangeoides Sieb. et Zucc.	1	0.3	30	0.04
Sum	365	100	73836	100

Chapter 1

A methodological survey on isolation and incubation of fungi on leaf litter

Introduction

Methodological development is important for ecological studies of decomposer fungi on leaf litter. As direct observation of fungal hyphae provides few information about identification, indirect methods that isolate fungi from incubated litter have been commonly used (Tokumasu 1980, 1982). The incubation is defined as the manipulation by which the germination of resting structures and the growth of hyphae are stimulated under suitable conditions of moisture and nutrients or enemy-free environment (Aoshima et al. 1983). The isolation is defined as the manipulation by which pure culture of fungi is obtained without contamination (Tubaki 1990).

In earlier works, successional changes in fungal populations on decomposing litters were investigated by the dilution plating method (Saito 1956; Ishii 1968; Deka and Mishra 1982; Kuter 1986; Singh et al. 1990). Most fungi isolated by this method are, however, derived from dormant spores attached to the litter surface (Warcup 1955; Christensen 1969). Hence, the relationship between litter decomposition processes and fungal succession described was unclear. Alternatively, to detect the function of fungal species in decomposition, a surface sterilization method that isolates fungi present within leaf tissues (Kinkel and Andrews 1988; Hata 1997) and a washing method that removes propagules on the surface and isolates actively growing mycelia (Harley and Waid 1955; Tokumasu 1980) have been developed and assured of their usefulness on several litter types (Kendrick and Burges 1962; Macauley and Thrower 1966; Tokumasu 1996, 1998a, 1998b).

Nutrient media and incubation period affect the isolation of fungi from litter (Aoshima et al. 1983; Tubaki 1990). For example, nutrient-rich media result in selective

isolation of fast-growing species, overlooking slow-growing species if present (Farrow 1954); short incubation period may overlook slow-growing and slow-sporulating species; on the other hand, contamination of fungi and mites may occur if the incubation period is too long. Tokumasu (1980) found that one-month incubation and weekly observation were successful for the observation of fungi on pine needle litter, but there have been few methodological survey on isolation and incubation of fungi on other leaf litter.

The present study adopted the surface sterilization method and the washing method for the description of mycobiota on beech leaves. In this chapter, a suitable methodology was explored for incubation of fungi on beech leaf litter by these methods. The effectiveness of surface sterilization and washing procedures was evaluated and the effect of nutrient media and incubation period on number of species and species composition is investigated.

Materials and Methods

Fungal isolation

A surface sterilization method (Kinkel and Andrews 1988; Hata 1997) and a modified washing method (Harley and Waid 1955; Tokumasu 1980) were used for the isolation of fungi from beech leaf litter. Leaf samples were collected from the Ashiu Experimental forest of Kyoto University (see Material and Study Site). Decomposing litters in litter bags (Chapter 4) collected on November 1997 (the 11th month) were used for surface sterilization method and on September 1997 (the 9th month) for washing method. The collected bags were placed in paper bags and taken to the laboratory. Fungal isolation was carried out within 8 hours after sampling. On each sampling occasion, a total of twenty leaf disks were punched from twenty leaves in the litter bags with a sterile cork borer (5.5 mm in diameter) from the central part of leaves, avoiding the primary vein.

For surface sterilization, leaf disks were submerged in 70% ethanol (v/v) for 1 min to wet the surface, then surface sterilized for 30 seconds in a solution of 15% hydrogen peroxide (v/v), and then submerged for 1 min in another 70% ethanol. The disks were rinsed with sterile distilled water, transferred to sterile filter paper in Petri dishes (9 cm in diameter), and dried for 24 h to suppress vigorous bacterial growth after plating (Widden and Parkinson 1973).

Imprints of leaf disks were made on LCA (Miura and Kudo 1970) and potato dextrose agar (PDA) for 3 leaf types: (i) surface sterilized disks treated with hydrogen peroxide; (ii) water control disks treated with sterile distilled water instead of hydrogen peroxide; and (iii) untreated control (Kinkel and Andrews 1988). Imprinted plates were incubated for one week at a room temperature. LCA and PDA were representative of nutrient-poor and nutrient-rich media, respectively. LCA contains glucose 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02%, and agar 1.3% (w/v). PDA (Nihon Suisan, Tokyo, Japan) contains glucose 2%, potato extract 0.4%, and agar 1.5% (w/v).

Fungi growing actively on the surface were isolated by the washing method. Leaf disks were washed in a sterile test tube using a vertical type shaker at 2000 rpm for 1.5 min. The disks were washed serially in five changes of 0.005% Aerosol-OT (Di-2-etylhexyl sodium sulfosuccinate) solution (w/v) and rinsed with sterile distilled water five times. The disks were transferred to sterile filter paper in Petri dishes (9 cm in diameter) and dried for 24 h to suppress vigorous bacterial growth after plating. The disks were then placed on 9 cm Petri dishes containing LCA or PDA, two disks per plate.

The petri dishes were sealed with parafilms (Hata 1997) and incubated at 20 °C in darkness for 8 weeks and observed at three days and at 6 to 9 day interval. Any fungal hyphae or spores appearing on the plates were transferred to fresh LCA agar plates for isolation and identification.

Number of colony forming unit (CFU) within the washing detergents was counted to ensure the effectiveness of spores removal from leaf surface by the washing method. The 1st, 4th, 7th, and 10th washings (1 ml) were mixed with 20 ml of molten malt agar (MA) medium (Hawksworth et al. 1995) in five Petri dishes. The plates were incubated for one week at 25°C in darkness. Any fungal colonies appearing on the plates were counted.

Results and discussion

Effectiveness of surface sterilization

Figure 1.1 shows leaf imprints of hydrogen peroxide treated, water control, and untreated disks. Hydrogen peroxide (15% solution, 30 seconds) was highly effective in eradicating fungi from leaf surfaces. An additional experiment indicated that longer immersion periods in the hydrogen peroxide excluded not only leaf surface microbes but also interior colonizers. This is especially the case for well decomposed litters as the sterilant can easily penetrate into porous litter structure. The immersion for 30 seconds is thus considered successful for the isolation of fungi from the inside of beech leaf litter.

Effectiveness of leaf washing

Table 1.1 shows number of colony forming unit (CFU) in one ml of washing detergents. The washing is regarded as effective when CFU in the washing is less than 10 (Tokumasu 1980). The number of CFU decreased as the washing to be less than 10 in the 10th washing. The result indicates that the washing procedure adopted in this study removed spores successfully from the litter surface.

Fig. 1.1 Imprints of hydrogen peroxide treated (left), water control (middle), and untreated (right) leaf disks on PDA (top row) and LCA (bottom row). Five imprints per plate.

Table 1.1 Number of colony forming unit (CFU) in one ml of washings

	1st	4th	7th	10th
Upper site	24.0	11.8	7.2	7.4
Lower site	43.6	11.8	10.4	8.4

Effect of medium and incubation period

Number of species isolated per leaf disk was 3.5±0.4 (mean ± s.e., n=20) on LCA (nutrient-poor medium) and 2.3±0.2 on PDA (nutrient-rich medium). The number of species was significantly higher on LCA than on PDA (p<0.01, t-test). The decrease of number of species on PDA is ascribed to the overgrowth of fast-growing species in *Mucor* and *Trichoderma* that suppress the growth and sporulation of less frequent, slow-growing species (Fig. 1.2). The result that number of species was higher in nutrient-poor medium than in nutrient-rich medium is consistent with Farrow (1954), Tokumasu (1980, 1982), and Kjøller and Struwe (1990). In addition, LCA effectively induces sporulation and is useful for identification (Tokumasu 1983). The LCA is thus successful for the isolation and observation of fungi on beech leaf litter.

Figure 1.3 shows cumulative number of species in relation to incubation period. The values were percentages of the cumulative number of species to the total number of species

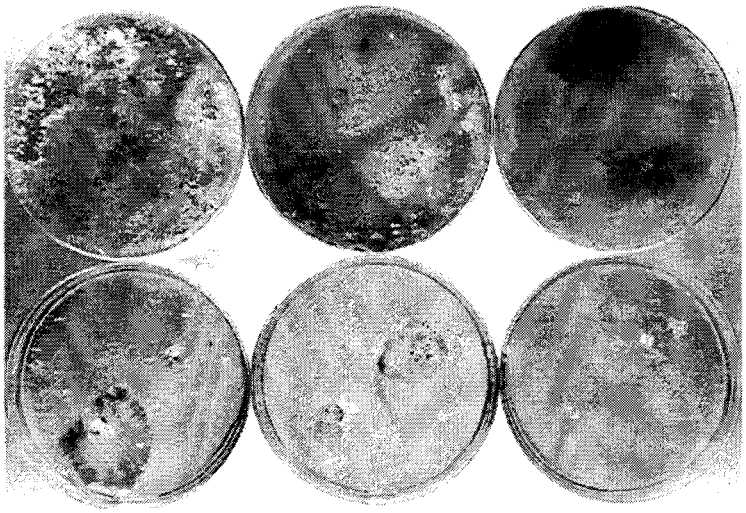

Fig. 1.2 Agar plates incubated for one month.

Top row = PDA, bottom row = LCA.

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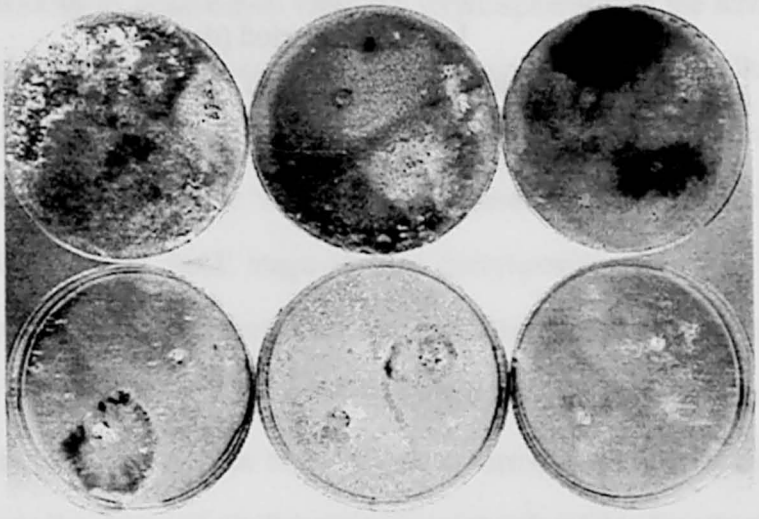

Fig. 1.2 Agar plates incubated for one month.

Top row = PDA, bottom row = LCA.

isolated during 2 month period. In the first week 24 (75%) of total 32 species and 16 (89%) of total 18 species were isolated on LCA and PDA, respectively. More than 90% of species were isolated in the third week on both media. The result indicated that two-month incubation was successful for the observation of fungi on beech leaf litter.

These results demonstrated the effectiveness of surface sterilization and washing methods for the isolation of fungi. Isolation on the nutrient-poor medium (LCA) for 2 months was proved successful for the incubation of fungi on beech leaf litter. In addition to nutrient media and incubation period, incubation temperature (Carreiro and Koske 1992) and sample size of inoculum (Bååth 1988) may also affect the number of species and deserve further investigation.

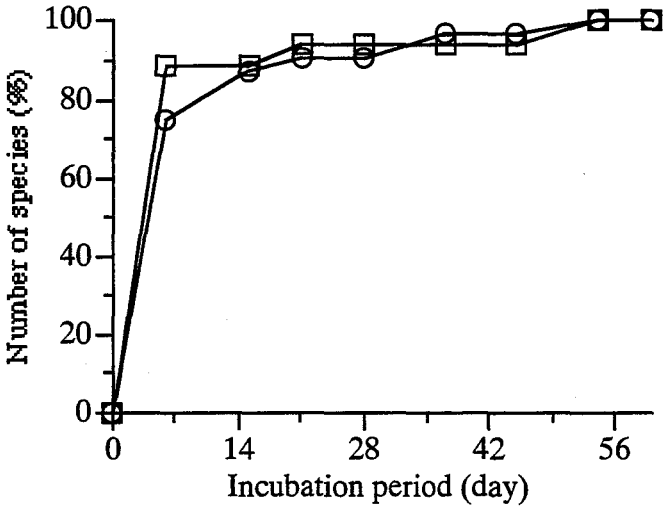

Fig. 1.3 Cumulative number of species in relation to incubation period. ○ = LCA, □ = PDA.

Chapter 2

Phyllosphere fungi on leaf litter: occurrence, colonization, succession, and bleaching

Introduction

The phyllosphere is the living leaf as a whole and includes the interior and surface (Carroll et al. 1977) and is colonized by a variety of micro-organisms (Fokkema and van den Heuvel 1986; Andrews and Hirano 1991). Phyllosphere fungi include endophytes and epiphytes that colonize the interior and surface of the phyllosphere, respectively, occupying two distinct habitats in the leaf (Petrini 1991). They have been studied intensively in terms of their ecological relationships with living plants (Clay 1987; Andrews and Hirano 1991; Carroll 1995). In contrast, the ecology of phyllosphere fungi on leaf litter has received little attention (Andrews 1991), even though they occur on various litters at initial stages of decomposition (Kendrick and Burges 1962; Dickinson 1965; Hogg and Hudson 1966; Ruscoe 1971a; Watson et al. 1974; Soma and Saito 1979; Wildman and Parkinson 1979; Mishra and Dickinson 1981; Cabral 1985; Kuter 1986; Stone 1987; Aoki et al. 1990, 1992; Osorio and Stephan 1991; Nakagiri et al. 1997; Okane et al. 1998; Müller et al. 2001).

Fungi persisting in dead leaves from the phyllosphere have the advantage of gaining access to readily available energy sources, such as non-lignified holocellulose and soluble carbohydrate abundant in freshly fallen leaves, prior to fungi that colonize after litter fall (Hudson 1968, 1971; Stone 1987). Thus, it has been considered that persistence is important for phyllosphere fungi during the initial stage of litter decomposition (Hudson 1968). However, some epiphytes have been isolated from sterilized leaf litter placed on litter layers (Tubaki and Yokoyama 1971, 1973a, 1973b; Kuter 1986), indicating that some phyllosphere fungi can colonize fallen litter directly by hyphae or spores from surrounding litter or the air. Experiments are needed to evaluate the ability of phyllosphere fungi to infect litter directly after litter fall.

Successional trends of phyllosphere fungi in decomposing leaf litter have been reviewed

by Hudson (1968). Primary saprophytes and parasites originating from the phyllosphere were considered to be important members in decomposer fungal associations on freshly fallen leaves but they disappeared as decomposition progressed. This general statement was largely based upon qualitative descriptive studies, and more detailed studies are needed to clarify the successional patterns of endophytes and epiphytes on leaf litter.

Phyllosphere fungi produce extracellular enzymes to utilize plant substrates; these enzymes are necessary not only for the colonization of living plant tissues but also for saprophytic growth in dead tissues (Hogg 1966; Hudson 1968, 1971; Carroll and Petrini 1983; Petrini et al. 1991; Sieber-Canavesi et al. 1991; White et al. 1991; Petrini et al. 1992; Bettucci and Saravay 1993). Xylariaceous endophytes in particular show a broad range of substrate utilization capabilities including ligninolytic and cellulolytic activity (Carroll and Petrini 1983) and are common in early decomposer communities in the sapwood of angiosperm trees (Boddy and Griffith 1989). Bleached litter in which brown litter color changed to bright yellow is a useful material to evaluate the possible effect of xylariaceous endophytes on litter decomposition, as the bleaching is associated with the decomposition of lignin and polyphenols (Hintikka 1970; Rihani et al. 1995; Osono and Takeda 1999b). Micromorphological observation of the bleached leaf tissues also provide useful information about the bleaching activity of endophytic fungi (Reisinger et al. 1978; Ponge 1991; Rihani et al. 2001).

This study examined the succession of phyllosphere fungi (endophytes and epiphytes) in living, senescent, freshly fallen, and decomposing leaves and the colonization of sterilized freshly fallen leaves by phyllosphere fungi to test their ability to infect litter directly after litter fall. Fungi were also isolated from the bleached and the non-bleached portions of the decomposing leaves. The chemical composition of the portions were then investigated to obtain the field evidence for lignocellulose decomposition by phyllosphere fungi. Japanese beech (*Fagus crenata* Blume) was chosen for the study because it has already been used in studies evaluating endophytic mycobiota and colonization of living leaves (Sahashi et al. 1999, 2000; Kaneko and Kakishima 2001) and in studies of the functional roles of litter decomposing fungi during the decomposition processes (Osono and Takeda 1999b, 2001a, 2001b, 2002a, 2002b).

Materials and methods

Sample collection

Samples were collected in the Ashiu Experimental Forest of Kyoto University (see Material and Study Site). Four types of beech leaves were collected at the study site during May 1999 - April 2000: living leaves, senescent leaves, freshly fallen leaves, and decomposing leaves.

Sixty living leaves were collected at monthly intervals from May to October 1999. On each sampling occasion, 10 leaves were cut from two branches at about 12 m height from each of two arbitrarily selected trees.

Thirty senescent leaves were collected on 4 November 1999 from two branches at about 12 m height from each of six arbitrarily selected trees.

One hundred and twenty freshly fallen leaves were collected on 2 November 1999 from the surface of the litter layer; 60 of these leaves were autoclaved at 120 °C for 20 min (denoted as sterilized leaves) and the remaining 60 were not sterilized (denoted as unsterilized leaves). The freshly fallen leaves were then placed on the litter layer on 4 November 1999. The 120 leaves were attached with pieces of vinyl thread to the petiole and attached to 10 metal pins (12 leaves per pin). A total of eighty of the leaves were retrieved at weekly intervals during November - December 1999 until snow fell, and another 40 leaves were retrieved on 28 April 2000 when snow had melted. Prior to the experiments, the sterilized leaves were placed on 2% malt extracted agar. After 8 weeks of incubation at 20°C, no microbial colonies had developed on the plates. Thus, the effectiveness of the sterilization method was assured.

Finally, 60 decomposing leaves were collected at monthly intervals from May to October 1999 at the same time as the living leaves. On each sampling occasion, 10 leaves were collected from the litter layer.

Fungal isolation

For the isolation of fungi, a surface sterilization method (Kinkel and Andrews 1988; Hata 1997) and a modified washing method (Harley and Waid 1955; Tokumasu 1980) were used.

Two leaf disks were punched from each single leaf with a sterile cork borer (5.5 mm in diameter) from the central part of leaves, avoiding the primary vein. One disk was used for the surface sterilization method and the other for the washing method. Fungal isolation was carried out within six hours of sampling. The methods are described in Chapter 1. The exception was that the disks from living and senescent leaves were washed serially in two changes of Aerosol-OT solution and rinsed with sterile distilled water four times. The surface sterilized and washed disks were placed on 9 cm Petri dishes containing LCA, one disk per plate.

Observation of bleached leaves

The number of bleached leaves and the bleached area present on litter were estimated on May to June 2001. All beech litters included in 10 cores (20 x 20 cm) that were arbitrarily placed on forest floor was collected and taken to the laboratory. Bleached leaves were photocopied and scanned with a photoscanner (EPSON GT-8000). By the image analysis performed on a Macintosh computer using public domain NIH image software (written by Wayne Rasband, US NIH. Email: < zippy.nimh.hih.gov>), area of bleached portions on the litter was measured (Osono and Takeda 1998).

Bleached leaves collected on August and September 1999 were cut into bleached and the non-bleached portions and used for fungal isolation and chemical analyses. Two bleached and two non-bleached leaf disks were punched out from each of twenty leaves and a total of 80 disks were used for fungal isolation by the surface sterilization and the washing methods as described above. Fungal isolation was carried out within six hours of sampling. Organic chemical and element contents of the bleached and the non-bleached portions were analyzed according to the method described below.

Bleached leaves collected on July 2002 were air-dried and prepared for thin sectioning. The leaves were embedded in polyethylene glycol #4000 (Nacalai Tesque Inc., Kyoto, Japan) which was heated to 80 °C and poured on the sample until it was no longer absorbed. The samples were then returned to a refrigerator at 4 °C to harden. The hardened samples were attached to a small wooden block (3 x 2 x 1 cm) using melted polyethylene glycol #4000 and sliced to the thickness of 30 μm by a sliding microtome (Daiwa Machinery Co., Saitama, Japan).

The thin sections of the leaves were then observed at 100 X magnification.

Chemical analyses

Leaf samples were ground in a laboratory mill (0.5 mm screen). The amount of lignin in samples was estimated by gravimetry using hot sulfuric acid digestion (King and Heath 1967). Each sample was extracted with alcohol-benzene at room temperature and the residue treated with 72% sulfuric acid (v/v) for 2 h at room temperature with occasional stirring. The mixture was then diluted with distilled water to make a 2.5% sulfuric acid solution and autoclaved at 120°C for 60 min. After cooling, the residue was filtered and washed with water through a porous crucible (G4), dried at 105°C, and weighed as acid insoluble residue. The filtrate (autoclaved sulfuric acid solution) was used for total carbohydrate analysis as described below.

The amount of total carbohydrate in the filtrate was estimated by the phenol-sulfuric acid method (Dubois et al. 1956). Five percent phenol (v/v) and 98% sulfuric acid (v/v) were added to the filtrate. The optical density of the solution was then measured by a spectrophotometer at 490 nm using known concentrations of D-glucose as standards.

Soluble carbohydrate and polyphenol were extracted from the sample with 50% methanol (v/v) at 75°C for 60 min. Soluble carbohydrate content was estimated by the phenol-sulfuric acid method. Polyphenol content was estimated by the Folin-Ciocalteau method (Waterman and Mole 1994). The extract was added with Folin-Ciocalteau reagent (Nacalai Tesque, Kyoto, Japan) and aqueous sodium carbonate. The optical density of the solution was then measured at 760 nm using the known concentrations of tannin acid as a standard. Holocellulose fraction was not determined by direct analysis but was calculated as a difference between the total carbohydrate and the soluble carbohydrate.

The term 'lignin' is commonly used for the material as determined by the sulfuric acid digestion method. 'Lignin' fraction contains not only true lignin but lignin-like materials (secondary compounds and humic substances) produced during decomposition. In this study the term "lignin" includes both substances for the sake of simplicity.

Total carbon and total nitrogen contents were estimated by automatic gas chromatography (NC analyzer, Sumitomo Chemical Co., Osaka, Japan).

Definition and data reduction

The frequency of all species was calculated as a percentage of the number of disks with the species of the total number of disks tested in each leaf type. When the frequency of a species on any leaf type was significantly ($P<0.05$) higher than zero by Fisher's exact probability test, the species was regarded as frequent. When comparing the frequency of a species between leaf types, Fisher's exact probability test on 2 x 2 contingency tables was used, because data were in the form of proportions.

In this study, phyllosphere denotes the interior and surface of living and senescent leaves. Fungi isolated from living, senescent, freshly fallen, and decomposing leaves were categorized into three groups: endophytes, epiphytes, and others. Endophytes were frequent species isolated from the phyllosphere by the surface sterilization method. Epiphytes were frequent species isolated from the phyllosphere by the washing method. The relative frequency of endophytes, epiphytes, and others was calculated as a percentage of the sum of frequencies of fungi in the group of the total frequency of all fungi in each leaf type.

Table 2.1 Frequency (%) of fungi in the interior of *Fagus crenata* leaves.

Fungus	Living leaves	Senescent leaves	Freshly fallen leaves		Decomposing leaves
			Unsterilized	Sterilized	
Geniculosporium sp.1	33.3	26.7	18.3	16.7	28.3
Ascochyta sp.	13.3	56.7	28.3	0.0	5.0
Xylaria sp. (anamorph)	10.0	10.0	6.7	6.7	33.3
White sterile 5LS12	6.7	26.7	6.7	3.3	10.0
White sterile STR	5.0	3.3	3.3	5.0	1.7
White sterile 8LS81	3.3	10.0	16.7	0.0	6.7
White sterile 7LS81	3.3	0.0	1.7	0.0	6.7
Coelomycete 7GS101	1.7	3.3	0.0	0.0	0.0
Nodulisporium sp.1	1.7	0.0	0.0	0.0	0.0
White sterile 7LS63	1.7	3.3	0.0	0.0	0.0
Ascomycete 8GS51	1.7	0.0	0.0	0.0	0.0
Aureobasidium pullulans	1.7	0.0	0.0	0.0	0.0
Coelomycete 10GS61	1.7	0.0	0.0	0.0	0.0
Coelomycete 7GS92	1.7	0.0	0.0	0.0	0.0
Discosia sp.	0.0	0.0	18.3	1.7	0.0
Ascomycete 8BS71	0.0	0.0	6.7	6.7	0.0
Dactylaria obtriangularia	0.0	3.3	10.0	3.3	0.0
Dark sterile DIM	0.0	0.0	13.3	0.0	1.7
Nodulisporium sp.3	0.0	20.0	0.0	1.7	1.7
Phoma sp.1	0.0	0.0	11.7	0.0	1.7
Eupenicillium sp.	0.0	0.0	1.7	5.0	1.7
Alternaria alternata	0.0	0.0	6.7	0.0	0.0
Geniculosporium sp.2	0.0	0.0	0.0	0.0	5.0
Arthrinium sp.	0.0	3.3	1.7	1.7	0.0
Discula sp.	0.0	0.0	5.0	0.0	0.0
Phomopsis sp.	0.0	0.0	3.3	0.0	0.0
Chaetomium globosum	0.0	0.0	1.7	0.0	3.3
Colletotrichum sp.1	0.0	3.3	1.7	0.0	0.0
White sterile 2CS63	0.0	0.0	3.3	0.0	0.0
Coelomycete 6CS51	0.0	0.0	1.7	1.7	0.0
Gliocladium roseum	0.0	0.0	1.7	0.0	1.7
Nodulisporium sp.2	0.0	0.0	0.0	1.7	1.7
White sterile 7LS63	0.0	0.0	0.0	1.7	1.7
Cladosporium tenuissimum	0.0	0.0	1.7	0.0	0.0
Coelomycete 3CS11	0.0	0.0	1.7	0.0	0.0

Table 2.1 Continued.

Coelomycete 3CS71	0.0	0.0	1.7	0.0	0.0
Hyphomycete 2CS72	0.0	0.0	1.7	0.0	0.0
Nigrospora state of *Khuskia oryzae*	0.0	0.0	1.7	0.0	0.0
Ulocladium sp.	0.0	0.0	1.7	0.0	0.0
Arthrinium phaeospermum	0.0	0.0	0.0	1.7	0.0
Cladosporium cladosporioides	0.0	0.0	0.0	1.7	0.0
Nigrospora sphaerica	0.0	0.0	0.0	1.7	0.0
Acrogenospora sp. (?)	0.0	0.0	0.0	0.0	1.7
Coelomycete 10LS71	0.0	0.0	0.0	0.0	1.7
Coelomycete 5LS51	0.0	0.0	0.0	0.0	1.7
Coelomycete WWF	0.0	0.0	0.0	0.0	1.7
Number of species	14	12	28	16	20

Table 2.2 Frequency (%) of fungi on the surface of *Fagus crenata* leaves.

Fungus	Living leaves	Senescent leaves	Freshly fallen leaves		Decomposing leaves
			Unsterilized	Sterilized	
Ascochyta sp.	60.0	76.7	16.7	10.0	3.3
Phoma sp.1	43.3	30.0	45.0	46.7	20.0
Cladosporium cladosporioides	30.0	53.3	26.7	20.0	15.0
Pestalotiopsis sp.1	23.3	6.7	0.0	0.0	0.0
Phomopsis sp.	20.0	0.0	1.7	0.0	0.0
Pestalotiopsis sp.3	15.0	16.7	13.3	3.3	11.7
Gliocladium roseum	13.3	46.7	16.7	15.0	51.7
Pestalotiopsis sp.2	10.0	60.0	20.0	23.3	11.7
Arthrinium sp.	8.3	6.7	33.3	30.0	15.0
Coelomycete WWF	8.3	0.0	0.0	0.0	0.0
Trichoderma viride	5.0	6.7	13.3	8.3	33.3
Unidentified BC	5.0	0.0	3.3	6.7	0.0
Aureobasidium pullulans	5.0	3.3	1.7	3.3	1.7
Pestalotiopsis sp.4	5.0	3.3	0.0	1.7	3.3
Alternaria alternata	3.3	20.0	21.7	3.3	3.3
Volutella ciliata	3.3	6.7	11.7	13.3	3.3
Penicillium thomii	3.3	0.0	3.3	1.7	10.0
Discula sp.	3.3	3.3	3.3	3.3	0.0
Coniothyrium sp.	3.3	0.0	1.7	3.3	0.0
Cladosporium sp.	3.3	0.0	1.7	1.7	0.0
Cladosporium sphaeospermum	3.3	3.3	0.0	0.0	0.0
Dark sterile 5GW72	3.3	0.0	0.0	0.0	0.0
White sterile 5GW21	3.3	0.0	0.0	0.0	0.0
Fusarium graminearum	1.7	0.0	3.3	6.7	6.7
Epicoccum nigrum	1.7	0.0	5.0	5.0	0.0
Paecilomyces sp.2	1.7	0.0	0.0	1.7	5.0
Paecilomyces sp.1	1.7	3.3	0.0	1.7	1.7
Penicillium oxalicum	1.7	0.0	0.0	0.0	1.7
Phialophora sp.	1.7	0.0	0.0	1.7	0.0
Coelomycete 3CS71	1.7	0.0	0.0	0.0	0.0
Coelomycete 5GW52	1.7	0.0	0.0	0.0	0.0
Curvularia lunata	1.7	0.0	0.0	0.0	0.0
Hyphomycete 7GW24	1.7	0.0	0.0	0.0	0.0
Trichoderma hamatum	0.0	0.0	16.7	15.0	43.3
Trichoderma koningii	0.0	0.0	8.3	6.7	33.3

Table 2.2 Continued.

Mortierella ramanniana var. *ramanniana*	0.0	0.0	5.0	3.3	30.0
Trichoderma sp.1	0.0	0.0	0.0	3.3	13.3
Penicillium citrinum	0.0	6.7	0.0	3.3	18.3
Mortierella isabellina	0.0	0.0	3.3	1.7	15.0
Mucor hiemalis	0.0	0.0	3.3	1.7	11.7
Mucor racemosus	0.0	0.0	0.0	1.7	15.0
Cladosporium herbarum	0.0	10.0	10.0	6.7	0.0
Dark sterile DIM	0.0	0.0	15.0	0.0	1.7
Mortierella globulifera	0.0	0.0	0.0	0.0	15.0
Trichoderma longibrachiatum	0.0	0.0	1.7	3.3	3.3
Fusarium solani	0.0	0.0	1.7	1.7	3.3
Coelomycete AKA	0.0	0.0	1.7	6.7	0.0
Phoma sp.2	0.0	3.3	3.3	1.7	1.7
Trichoderma atroviride	0.0	3.3	0.0	0.0	6.7
Trichoderma sp.2	0.0	0.0	1.7	1.7	5.0
Absidia glauca	0.0	0.0	0.0	0.0	8.3
Mortierella verticillata	0.0	0.0	0.0	0.0	8.3
Penicillium miczynskii	0.0	0.0	0.0	0.0	3.3
Verticillium psalliotae	0.0	0.0	1.7	0.0	3.3
Trichoderma polysporum	0.0	0.0	0.0	1.7	5.0
Penicillium velutinum	0.0	0.0	0.0	0.0	3.3
Gliocladium virens	0.0	0.0	0.0	0.0	3.3
Acremonium sp.	0.0	10.0	0.0	0.0	1.7
Penicillium sclerotiorum	0.0	3.3	3.3	0.0	1.7
Penicillium glabrum	0.0	0.0	3.3	0.0	0.0
Dactylaria obtriangularia	0.0	0.0	1.7	5.0	0.0
Verticillium cf. *suchlasporium*	0.0	0.0	1.7	0.0	1.7
Nigrospora state of *K. oryzae*	0.0	10.0	0.0	0.0	0.0
Monochaetia sp.	0.0	3.3	0.0	3.3	0.0
White sterile 3SW91	0.0	0.0	0.0	5.0	0.0
Mucor sp.	0.0	0.0	0.0	0.0	1.7
Geniculosporium sp.3	0.0	6.7	0.0	0.0	0.0
Colletotrichum sp.1	0.0	3.0	0.0	1.7	0.0
Cladosporium oxysporum	0.0	3.0	0.0	0.0	1.7
Mortierella sp.1	0.0	0.0	1.7	1.7	0.0
White sterile 5SW61	0.0	0.0	1.7	1.7	0.0
Trichoderma harzianum	0.0	0.0	1.7	0.0	0.0

Table 2.2 Continued.

Mortierella wuyshanensis	0.0	0.0	1.7	0.0	1.7
Nigrospora sphaerica	0.0	0.0	0.0	1.7	1.7
Penicillium janthinellum	0.0	0.0	0.0	0.0	1.7
Mucor circinelloides	0.0	0.0	0.0	0.0	3.3
Geniculosporium sp.1	0.0	3.3	0.0	0.0	0.0
Penicillium sp.	0.0	3.3	0.0	0.0	0.0
Pestalotiopsis sp.5	0.0	3.3	0.0	0.0	0.0
Cladosporium macrocarpum	0.0	0.0	1.7	0.0	0.0
Colletotrichum sp.2	0.0	0.0	1.7	0.0	0.0
Fusarium avenaceum	0.0	0.0	1.7	0.0	0.0
Fusarium oxysporum	0.0	0.0	1.7	0.0	0.0
Hyphomycete 3CW24	0.0	0.0	1.7	0.0	0.0
Hyphomycete 4CW31	0.0	0.0	1.7	0.0	0.0
Phialophora verrucosa	0.0	0.0	1.7	0.0	0.0
Trichoderma piluliferum	0.0	0.0	1.7	0.0	0.0
Ulocladium sp.	0.0	0.0	1.7	0.0	0.0
Coelomycete 1SW42	0.0	0.0	0.0	1.7	0.0
Coelomycete 4SW26	0.0	0.0	0.0	1.7	0.0
Cylindrocarpon state of *Nectria radicicola*	0.0	0.0	0.0	1.7	0.0
Phialophora cycalminis	0.0	0.0	0.0	1.7	0.0
Calcarisporium arbuscula	0.0	0.0	0.0	0.0	1.7
Eupenicillium sp.	0.0	0.0	0.0	0.0	1.7
Mortierella alpina	0.0	0.0	0.0	0.0	1.7
Number of species	33	30	48	48	52

Table 2.3 Frequency (%) of phyllosphere fungi in the interior and on the surface of living, senescent, freshly fallen (unsterilized), and decomposing leaves of *Fagus crenata*. These fungi were divided into three groups according to the frequency on freshly fallen and decomposing leaves (see text).

Fungus	Endophyte / epiphyte	Group	Living leaves	Senescent leaves	Freshly fallen leaves	Decomposing leaves
Interior						
Geniculosporium sp.1	endophyte	I	33	27	18	28
Xylaria sp. (anamorph)	endophyte	I	10	10	7	33
White sterile mycelium 5LS12	endophyte	I	7	27	7	10
Ascochyta sp.	endophyte/epiphyte	II	13	57	28	5
Nodulisporium sp.3	endophyte	III	0	20	0	2
Phoma sp.1	epiphyte	*	0	0	12	2
Surface						
Phoma sp.1	epiphyte	I	43	30	45	20
Cladosporium cladosporioides	epiphyte	I	30	53	27	15
Pestalotiopsis sp.3	epiphyte	I	15	17	13	12
Gliocladium roseum	epiphyte	I	13	47	17	52
Pestalotiopsis sp.2	epiphyte	I	10	60	20	12
Arthrinium sp.	epiphyte	I	8	7	33	15
Ascochyta sp.	endophyte/epiphyte	II	60	77	17	3
Alternaria alternata	epiphyte	II	3	20	22	3
Pestalotiopsis sp.1	epiphyte	III	23	7	0	0
Phomopsis sp.	epiphyte	III	20	0	2	0
Coelomycete WWF	epiphyte	III	8	0	0	0

* *Phoma* sp.1 was an epiphytic species in Group I but was also frequent in the interior of freshly fallen leaves.

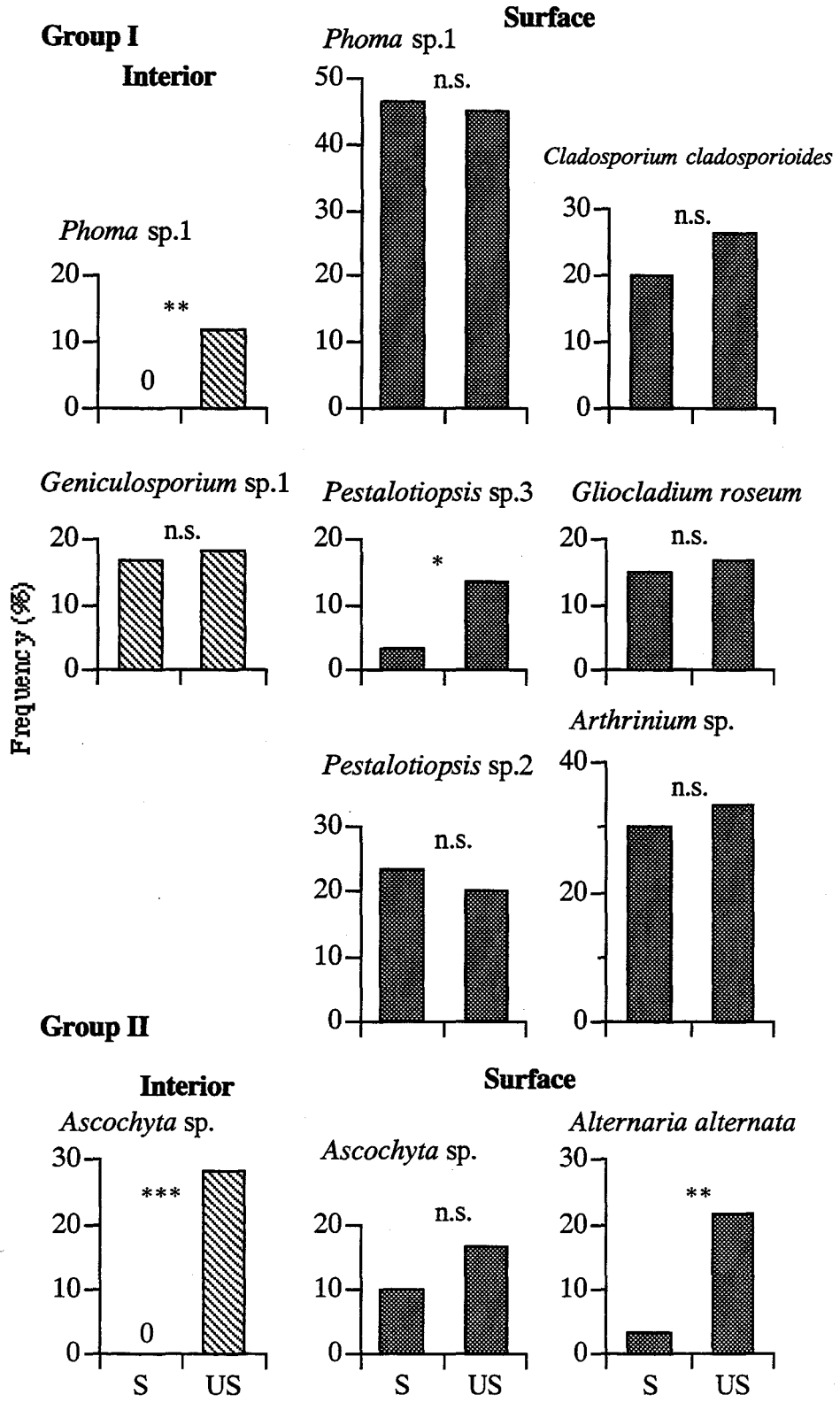

Fig. 2.1 Frequency of phyllosphere fungi in the interior and on the surface of sterilized (S) and unsterilized (US) freshly fallen leaves of *Fagus crenata*. *** = $P<0.001$; ** = $P<0.01$; * = $P<0.05$; n.s. = non significant.

Results

Phyllosphere fungi and their occurrence on leaf litter

Eighteen fungal species were isolated from the interior and 47 from the surface of the phyllosphere (living and senescent leaves) of beech (Tables 2.1 and 2.2).

Fifteen species were recorded as phyllosphere fungi (Table 2.3). Five species in the interior were regarded as endophytes and eleven species on the surface were regarded as epiphytes. *Ascochyta* sp. was frequent in both habitats. These phyllosphere fungi were divided into three groups according to their frequency on freshly fallen and decomposing leaves. Group I included nine species (three endophytes and six epiphytes) that were frequent on decomposing leaves or on both freshly fallen and decomposing leaves. *Phoma* sp.1 was an epiphyte but was also frequent in the interior of freshly fallen leaves. Group II included two species, *Ascochyta* sp. and *Alternaria alternata*, which were frequent in freshly fallen leaves but not so on decomposing leaves. Group III included four species (one endophyte and three epiphytes) that were not frequent on either freshly fallen or decomposing leaves.

Colonization of sterilized litter by phyllosphere fungi

Figure 2.1 shows frequency of 10 phyllosphere fungi belonging to Groups I and II on sterilized freshly fallen leaves in comparison with unsterilized leaves. The results for *Xylaria* sp. (anamorph) and a white sterile mycelium coded 5LS12 in Group I and four species in Group III were excluded from Fig. 2.1, as they were not frequent on freshly fallen leaves. The frequencies of *Ascochyta* sp. and *Phoma* sp.1 in the interior were significantly lower on sterilized leaves than on unsterilized leaves, but on the surface the differences were not significant. The frequencies of *Alt. alternata* and *Pestalotiopsis* sp.3 on the surface were significantly lower on sterilized leaves than on unsterilized leaves.

Succession of phyllosphere fungi on leaf litter

Figure 2.2 shows sum of frequencies, the relative frequency, and number of species

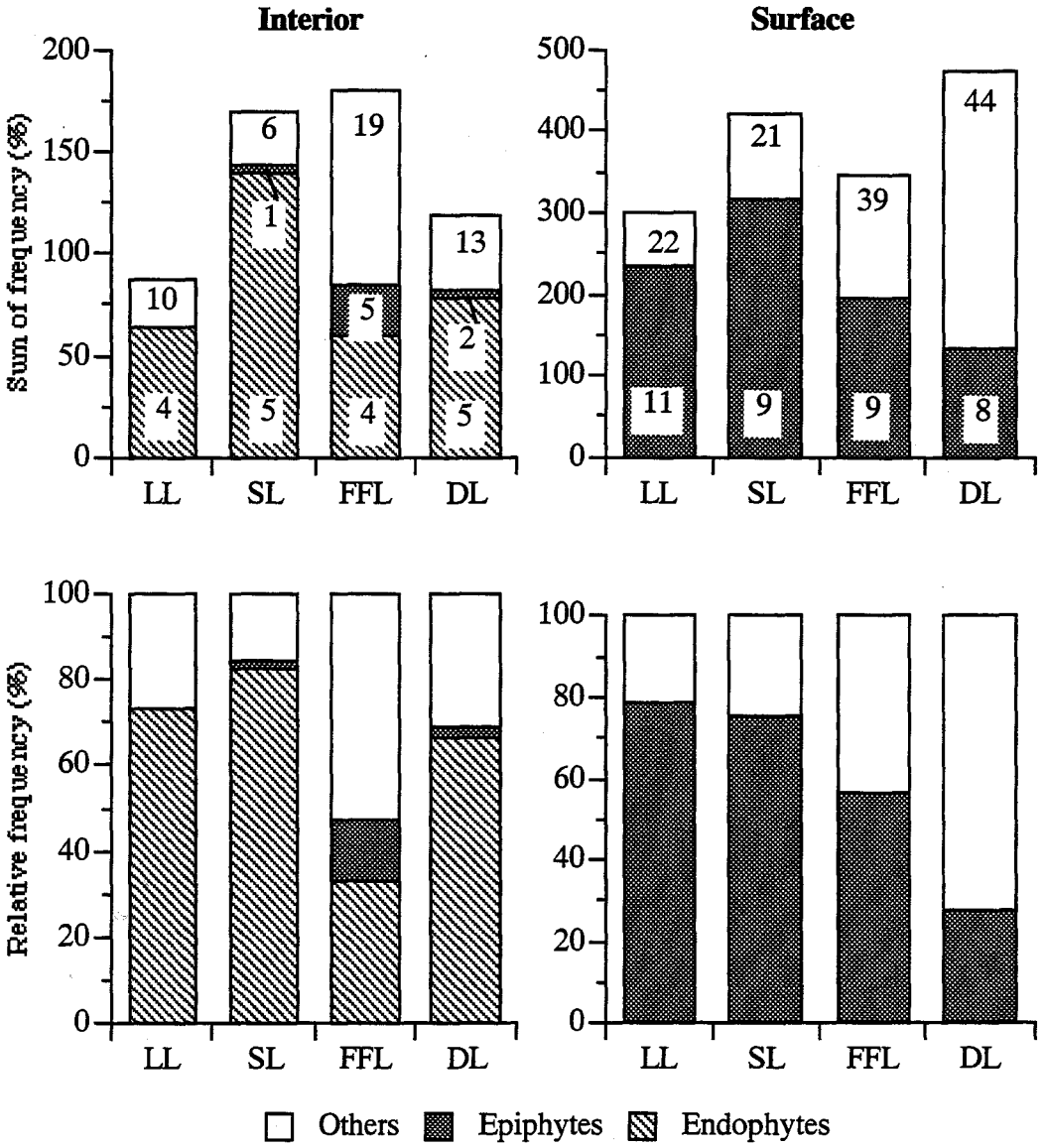

Fig. 2.2 Sum of frequencies, relative frequencies, and numbers of species of endophytes, epiphytes, and other species in the interior and on the surface of living (LL), senescent (SL), freshly fallen (unsterilized) (FFL), and decomposing leaves (DL) of *Fagus crenata*. Values indicate numbers of species.

of endophytes, epiphytes, and other species on the interior and surface of living, senescent, freshly fallen (unsterilized), and decomposing leaves. Endophytes as a group accounted for 73% and 84% of the total frequency in the interior of living and senescent leaves, respectively. The sum of frequencies and the relative frequency of endophytes decreased temporarily on freshly

fallen leaves while the sum of frequencies and the relative frequency of epiphytes and other species increased. The sum of frequencies and the relative frequency of endophytes then increased on decomposing leaves while the sum of frequencies and the relative frequency of epiphytes and other species decreased.

Epiphytes as a group accounted for 79% and 75% of the total frequency on the surface of living and senescent leaves, respectively. The sum of frequencies and the relative frequency of epiphytes decreased as decomposition progressed from freshly fallen to decomposing leaves while the sum of frequencies and the relative frequency of other species increased.

Bleached leaf litter

Four hundred and twenty two (80%) of 530 leaves examined suffered bleaching (an example of bleached leaf litter is shown in Fig. 2.3). However, the bleached portion consisted of 6.3% of the total leaf area on mean with the standard deviation of 5.1% (n=10).

A total of 39 isolates in 8 species were isolated from the interior and a total of 186 isolates in 43 species were isolated from the surface of the bleached and the non-bleached portions (Tables 2.4 and 2.5). Two xylariaceous species *Xylaria* sp. (anamorph) and *Geniculosporium* sp.1 were frequent in the interior of both portions (Table 2.4). The frequency of *Xylaria* sp. (anamorph) was significantly higher in the bleached portion than in the non-bleached portion, while no significant differences were found in the frequencies of the other species in the interior and on the surface between the portions.

Fig. 2.3 A bleached leaf litter of *Fagus crenata* collected from the field.

Table 2.4 Frequency of occurrence (%) of fungi isolated by surface sterilization method from bleached and non-bleached portions of *Fagus crenata* leaf litter. n.s. = no significant.

Fungus	Bleached portion	Non-bleached portion	P
Xylaria sp. (anamorph)	60	20	0.01
Geniculosporium sp.1	25	35	n.s.
White sterile 5LS12	15	10	n.s.
Geniculosporium sp.2	5	0	n.s.
Hyphomycete 8BS31	0	10	n.s.
Ascomycete 8BS71	0	5	n.s.
Nodulisporium sp.1	0	5	n.s.
Phomopsis sp.	0	5	n.s.
Number of species	4	7	

Table 2.5 Frequency of occurrence (%) of fungi isolated by washing method from bleached and non-bleached portions of *Fagus crenata* leaf litter. n.s. = no significant.

Fungus	Bleached portion	Non-bleached portion	P
Trichoderma sp.1	40	60	n.s.
Gliocladium roseum	35	40	n.s.
Trichoderma viride	25	45	n.s.
Trichoderma hamatum	25	35	n.s.
Mucor hiemalis	25	20	n.s.
Mortierella ramanniana	20	35	n.s.
Cladosporium cladosporioides	20	20	n.s.
Mucor racemosus	20	20	n.s.
Mortierella isabellina	15	40	n.s.
Penicillium citrinum	15	30	n.s.
Arthrinium sp.[a)]	15	25	n.s.
Trichoderma koningii	15	0	n.s.
Coelomycete 8WW71	10	5	n.s.
Penicillium glabrum	10	0	n.s.
Verticillium psalliotae	10	0	n.s.
Penicillium velutinum	5	10	n.s.
Pestalotiopsis sp.3	5	10	n.s.
Dark sterile DIM	5	5	n.s.
Mortierella globurifera	5	5	n.s.
Mucor sp.	5	5	n.s.
Pestalotiopsis sp.2	5	5	n.s.

Table 2.5 Continued.

Trichoderma longibrachiatum	5	5	n.s.
Chaetomium globosum	5	0	n.s.
Epicoccum nigrum	5	0	n.s.
Gliocladium viride	5	0	n.s.
Mortierella sp.	5	0	n.s.
Penicillium janthinellum	5	0	n.s.
Phoma sp.	5	0	n.s.
Trichoderma atroviride	5	0	n.s.
Trichoderma sp.2	5	0	n.s.
Penicillium miczynskii	0	20	n.s.
Fusarium solani	0	15	n.s.
Gliocladium virens	0	15	n.s.
Penicillium thomii	0	15	n.s.
Absidia spinosa	0	10	n.s.
Colomycete AKA	0	10	n.s.
Paecilomyces carneus	0	10	n.s.
Verticillium cf. *suchlasporium*	0	10	n.s.
Volutella ciliata	0	10	n.s.
Absidia glauca	0	5	n.s.
Mortierella verticillata	0	5	n.s.
Trichoderma harzianum	0	5	n.s.
Trichoderma polysporum	0	5	n.s.
Number of species	30	32	

a) Anamorphic state of *Apiospora montagnei.*

Table 2.5 shows organic chemical and element contents in the bleached and the non-bleached portions. In the bleached portion, lignin and polyphenol concentrations were lower, while holocellulose and soluble carbohydrate concentrations were higher, than in the non-bleached portion. Carbon concentration was lower while nitrogen concentration was higher in the bleached portion than in the non-bleached portion.

Figure 2.4 shows section of freshly fallen leaves and bleached and non-bleached portions of decomposing leaves. In the bleached portions, intracellular brown pigmentation was cleared and cell walls were thinned in palisade and spongy tissues.

Table 2.5 Organic chemical and element concentration (%) of bleached and non-bleached portions of *Fagus crenata* leaf litter

Property	Bleached portion	Non-bleached portion
Organic chemical		
Lignin	34.8	41.8
Holocellulose	30.2	25.2
Polyphenol	0.7	1.1
Soluble carbohydrate	2.9	2.6
Element		
Carbon	44.9	46.5
Nitrogen	2.3	2.1

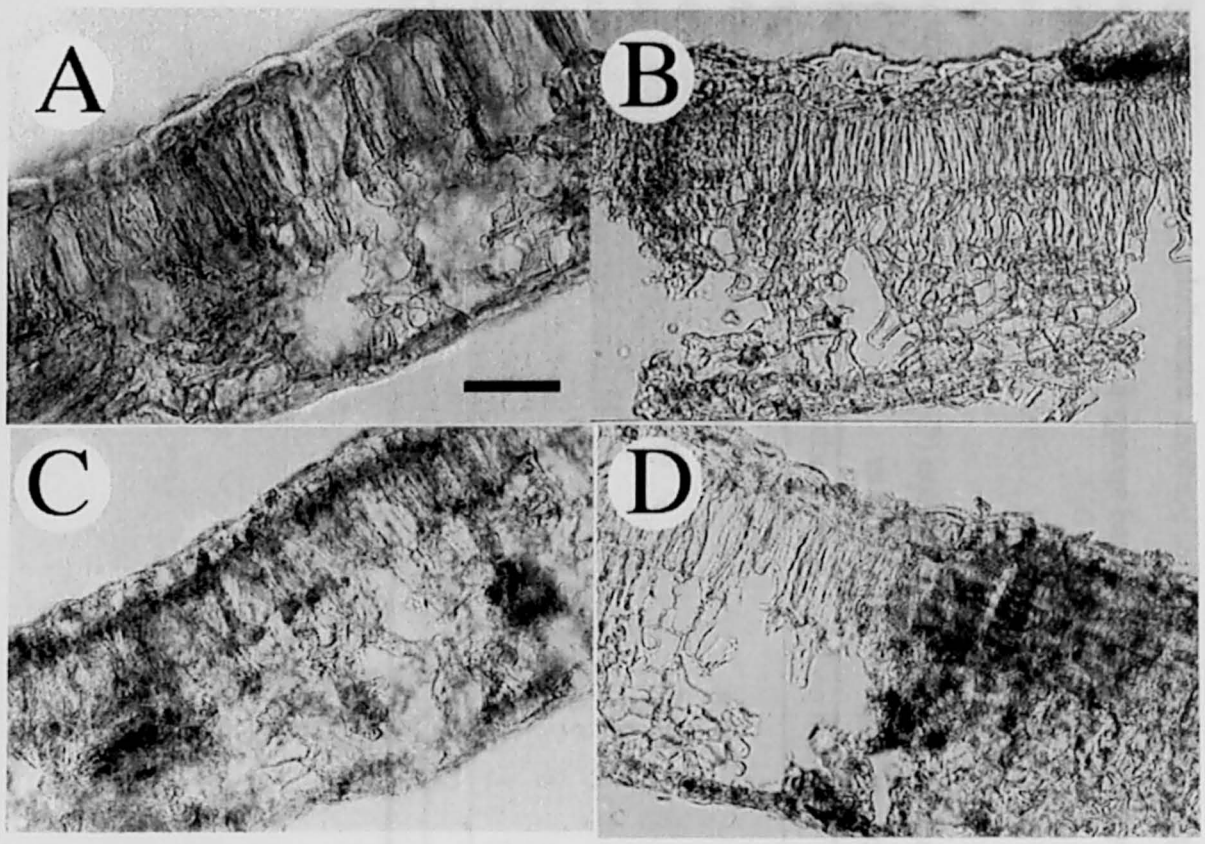

Fig. 2.4 Vertical thin sections of beech leaf litter. A = freshly fallen leaves, B = bleached portion of decomposing leaves, C = non-bleached portion of decomposing leaves, D = bleached and non-bleached portion. Bar indicates 30 μm

Discussion

Of the fifteen species recorded as phyllosphere fungi, eleven occurred frequently on freshly fallen and (or) decomposing leaves. Xylariaceous endophytes were predicted to occur in leaf litter (Carroll and Petrini 1983; Petrini and Petrini 1985), but only two previous studies found circumstantial evidence to support this (Bills and Polishook 1994; Laessøe and Lodge 1994). The present study is therefore the first to demonstrate the frequent occurrence of xylariaceous endophytes, *Geniculosporium* sp.1, *Xylaria* sp. (anamorph), and a white sterile 5LS12, on leaf litter. Preliminary DNA analysis indicated 5LS12 also belongs to the Xylariaceae.

The frequencies of *Alt. alternata* and *Ascochyta* sp. classified in Group II were low on decomposing leaves compared to the frequencies of fungi classified in Group I. This difference may be explained by differences in competitive abilities, life history characteristics, or energy requirements between fungi classified in Groups I and II. The latter explanation is probable as previous studies indicated that freshly fallen leaves are richer in readily available energy sources such as non-lignified holocellulose and soluble carbohydrate than are decomposing leaves (Osono and Takeda 2001b) and that these fungi in Group II had a higher requirement for these sources (Osono and Takeda 2002a).

Four species classified in Group III that were infrequent on freshly fallen and decomposing leaves may be non saprophytic; these fungi may fail to develop mycelia and be excluded at leaf death.

Phyllosphere fungi may persist in leaf litter from the phyllosphere and (or) infect leaves directly after litter fall. In this study, colonization of sterilized freshly fallen leaves was investigated to test their ability to infect litter directly after litter fall. The decrease in frequencies of *Ascochyta* sp. and *Phoma* sp.1 in the interior and *Alt. alternata* and *Pestalotiopsis* sp.3 on the surface of sterilized leaves suggested that persistence was more important than direct infection for these species. The frequent occurrence of *Ascochyta* sp. and *Phoma* sp.1 on the surface of sterilized leaves indicated that habitat differences between the interior and surface affected the infection processes of these species; direct infection to the surface was intensive but persistence in the interior was a crucial process. The dependence upon persistence of *Alt. alternata* and

Ascochyta sp. in Group II may be related to their high requirement for readily available resources in freshly fallen leaves as discussed above, suggesting that the infection strategy of phyllosphere fungi would be associated with the degree of energy requirement for each species.

On the other hand, the frequencies of *Arthrinium* sp., *Geniculosporium* sp.1, *C. cladosporioides*, *Gl. roseum*, and *Pestalotiopsis* sp.2 did not differ significantly between sterilized and unsterilized leaves, indicating these fungi were able to infect litter directly. Tubaki and Yokoyama (1971, 1973a, 1973b) and Kuter (1986) also reported high densities of *Cladosporium* and *Gliocladium* species on sterilized leaf litter as well as on unsterilized litter. Colonization of sterilized litter suggested that these phyllosphere fungi can infect fallen litter directly by hyphae or spores from surrounding litter or the air. Immigration may play a significant role in the population dynamics of some phyllosphere fungi on freshly fallen leaves. Similarly, Kinkel et al. (1989) and Kinkel (1991) found in a study of fungal colonization of surface-sterilized living leaves that immigration was quantitatively important in the population processes of phylloplane fungi.

Analyses of the sum of frequencies and the relative frequency of endophytes, epiphytes, and other species revealed successional trends during decomposition from freshly fallen to decomposing leaves. The temporary decrease in frequency of endophytes on freshly fallen leaves may be due to competition with epiphytes and other species for readily available energy sources in the mesophyll. A similar situation was found on leaves of *Eucalyptus viminalis* Labill., where, at the time of leaf death, epiphytes colonized the interior while the frequency of the original endophytes decreased (Cabral 1985). The decrease in epiphytes in the interior of decomposing leaves is probably due to the consumption and exhaustion of readily available resources of plant origin during decomposition (Osono and Takeda 2002a). The ability of the xylariaceous endophytes in Group I to decompose the residual lignocellulose matrix may allow them to persist in decomposing leaves (Osono and Takeda 2001b, 2002a). On the other hand, the sum of frequencies and the relative frequency of epiphytes on the surface decreased as decomposition progressed from freshly fallen to decomposing leaves. Osono and Takeda (2001b) found that the decrease in the frequencies of epiphytes during decomposition was related to the decrease in readily available resources on which they depend for their growth. The successional pattern

observed on the surface of beech litter was similar to that reviewed by Hudson (1968) on decomposing litter of several tree species.

Xylaria sp. (anamorph) and *Geniculosporium* sp.1 were frequent in the interior of the bleached portions in which cell walls were thinned and intracellular brown pigments were removed in palisade and spongy tissues and in which lignin concentration was lower than in the non-bleached portion. The removal of cell wall components and condensed protoplasmic residues by white rot fungi has already reported by Reisinger et al. (1978) and Rihani et al. (2001). As these xylariaceous bleached the litter and decomposed lignin in pure culture test (Osono and Takeda 2002a), these fungi took part in the selective delignification in the bleached portion under field condition. Furthermore, the frequency of *Xylaria* sp. (anamorph) was significantly higher in the bleached portion than in the non-bleached portion, indicating that some strains of *Xylaria* sp. (anamorph) had a prominent role in lignin decomposition of the litter. Osono and Takeda (2002a) found a strain of this species caused marked delignification in pure culture decomposition test.

Xylaria sp. (anamorph) decomposed not only lignin but also holocellulose to some extent during the selective delignification process. This was due to that lignin decomposition depends on the availability of carbohydrate energy sources (Kirk et al. 1976). Higher concentration of soluble carbohydrate in the bleached portion may be due to the release of reducing sugars from the holocellulose fraction during the selective delignification (Saito 1960). The bleached portions, however, consisted of only a small part of the total leaf area (6.3%) and the majority of the leaves was the non-bleached portions in which holocellulose was preferentially attacked over lignin. This is consistent with the result of Osono and Takeda (2001b) that reported these xylariaceous fungi were frequent during the immobilization phase of nitrogen and phosphorus in which holocellulose was decomposed preferentially over lignin.

The roles of phyllosphere fungi in litter decomposition have been investigated previously (Osono and Takeda 1999*b*, 2001a, 2001b, 2002a). Endophytes have different functions than epiphytes: xylariaceous endophytes *Geniculosporium* sp.1, *Xylaria* sp. (anamorph), and 5LS12 are functional species decomposing the lignocellulose matrix and regulating nutrient dynamics, whereas epiphytes and other endophytes are associated species

whose growth depends upon holocellulose and (or) soluble carbohydrate of plant origin. The absence of basidiomycetous fungi on freshly fallen and decomposing leaves is probably due to the isolation methodology adopted in this study. Fungal biomass estimation by a direct observation technique revealed that biomass of the Basidiomycota was negligible on freshly fallen leaves and increased as decomposition progressed (Osono and Takeda 2001b).

Chapter 3

Species composition and abundance of litter-decomposing Basidiomycota with reference to bleaching activity

Introduction

In forest ecosystems, the Basidiomycota have central roles in litter decomposition and in symbiotic uptake of plant nutrients (Frankland et al. 1982; Cooke and Rayner 1984; Smith and Read 1997). Hyphae of these fungi are distinguished from those of others based on the presence of clamp connection at septa. Mycelia of the Basidiomycota (as clamp bearing hyphae) consist of up to 70% of total fungal mycelium in forest soil (Frankland 1982; Kjøller and Struwe 1982). However, observations of hyphae gave no information about the identification of species. Hence, species composition has been investigated from observation of fruit bodies or molecular analyses of the hyphae.

Ecology of litter decomposing Basidiomycota has been studied from several aspects including species composition (Hering 1966; Okabe 1986; Miyamoto et al. 2000), vertical and horizontal distribution of mycelia on forest floors (Swift 1982; Newell 1984a, b), autecology (Frankland 1984; Frankland et al. 1995), population dynamics (Murphy and Miller 1993, 1997), colonization of decomposing litter (Osono and Takeda 2001b), degradation of lignin and humic acid and ligninolytic enzymatic activity (Blondeau 1989; Steffen et al. 2000), and litter decomposing activity *in vitro* (Lindeberg 1944, 1946, Mikola 1956, Saito 1960, Hering 1967, 1972, De-Boois 1976, Dix and Simpson 1984, Kuyper and Bokeloh 1994, Osono and Takeda 1999b, 2002a) and *in vivo* (Harris 1945; Saito 1957; Hintikka 1970). These aspects were studied separately and a pilot study that follows these aspects simultaneously is needed to clarify the role of the Basidiomycota in litter decomposition.

The purpose is to investigate species composition and mycelial abundance of the Basidiomycota with reference to bleaching activity of these fungi. The bleaching of litter is analogous to white rot of wood that is due to removal of lignin as well as cellulose.

Materials and Methods

Study area

The study was carried out in the Ashiu Experimental Forest of Kyoto University (see Material and Study Site). Two study sites were chosen that were located on a west facing slope about 200 m long. One site was located on the upper part of the slope and the other on the lower part. A study plot of 50 x 10 m in area (500 m^2) was laid out in each site and was divided into 5 subplots of 10 x 10 m. The study area of 500 m^2 was enough to describe species composition of macrofungi in temperate forests according to Okabe (1986). The subplots were divided into 25 grids of 2 x 2 m to make a total of 125 grids per site.

Fruit body collection

Fruit body of the Basidiomycota was collected from the plots 9 times during May to November 2001. At each sampling, all fruit bodies encountered on the surface of forest floor were recorded excepting obviously immature or rotting ones. Records kept of species and of grid number and soil horizons (L, F, A layer) from which the fruit body was emerged. Fruit bodies occurring on logs, twigs, or roots that were fallen or buried were not recorded. Ascomycota were omitted. Identification was mainly made after Imazeki et al. (1988), Imazeki and Hongo (1987, 1989), and Hongo (1994). The fungi were divided into two groups, litter decomposing fungi and mycorrhizal fungi, according to these publications. Frequency of a species was calculated as a percentage of the number of grids with the species to the total number of grids examined in each site (125).

Collection of forest floor and mineral soil materials

Forest floor and mineral soil materials were collected from each of 10 subplots four times on May, July, September, and November 2001 and used for fungal biomass estimation. Materials from L, F, and A layers were collected using a core of 20 x 20 cm. The materials

were preserved in vinyl bags and taken to the laboratory. L layer materials on November were divided into two portions, freshly fallen litter and previously partly decomposed litter. F layer materials on the lower site were sampled only when these were recognized, as the development of F layer was poor on the lower site (Takeda and Kaneko 1988). Samples were preserved in a refrigerator at 4°C until use and processed within 48 h after sampling. L layer materials were fragmented by a blender, and F and A layer materials were passed through a 2 mm sieve to exclude plant roots and coarse fragments, before a portion of the sample was used for the homogenization.

Fungal biomass estimation

Fungal biomass was measured by the method of Jones and Mollison (1948) as modified by Ono (1998). Samples of 1 g (fresh weight) were homogenized in a blender at 10000 rev min^{-1} in 49 ml of distilled water for 3 min. The suspension (20 ml) was diluted with 20 ml of molten agar solution (final concentration 1.5%) and mixed at low speed on a magnetic stirring plate. Agar films were prepared from each suspension in a haemocytometer (0.1 mm depth), transferred to glass slides, and dried for 24 hours.

The films were dual-stained with fluorescent brightener (FB) and acridine orange (AO), each for 1 h. FB binds to chitin in fungal cell walls (West 1988) to visualize all hyaline hyphae that are live or ghost (empty). AO binds to nucleic acids in live fungal cells (Rost 1992) to visualize live hyphae. The dual staining method was used because this method allows both total (live plus empty) hyphal length and live hyphal length to be counted in the same microscopic field to estimate the proportion of live hyphal length to total hyphal length accurately. Three agar films were prepared for each sample.

The stained films were mounted between slides and coverslips with one drop of immersion oil (type DF, Cargille Laboratories, inc., Cedar Grove, N.J., USA) and examined with a Nikon Microphot-SA epifluorescent microscope equipped with a high-intensity mercury light source. A Nikon UV-1A filter cube was used for examination of FB-stained

hyphae, and a Nikon B-2H filter cube was used for AO-stained hyphae. Dark-pigmented hyphae that were not stained with FB, were observed by bright field microscopy. Microscope fields were selected randomly and 25 fields were observed for each slide at 1000 X magnification. This magnification yields larger values for hyphal length than magnifications below 1000 X (Newell 1992). Hyphal lengths were estimated using an eye-piece grid and a grid-intersection method (Olson 1950) and were scored into six nominal diameter classes (<0.8, 0.8-2.4, 2.4-4.0, 4.0-5.6, 5.6-7.2, and >7.2 μm). Fungal biomass was calculated using a measured mean hyphal length and diameter for each subsample at each sampling occasion, considering fungal hyphae to be cylinders. A density of 1.1 g cm^{-3} (Saito 1955) and a dry weight content of 15% (Bååth and Söderström 1977) were used.

Total fungal biomass was calculated as the sum of FB-stained biomass and the dark-pigmented biomass. Hyphae with clamp connections were classified into the Basidiomycota. It is recognized, however, that the fungal biomass of the Basidiomycota may have been underestimated because the frequency of clamp formation varies between species. AO-stained hyphae were regarded as living (Ono 1998). Separate litter samples were dried at 105°C for one week to determine water content and convert fresh weight to dry weight.

Bleached litter

Litter materials that suffered bleaching by basidiomycetous fungi were qualitatively collected from forest floors of the study area. These materials are denoted as 'bleached litter'. At the same time, litter materials surrounding the bleached litter but suffered no bleaching were collected as a control and denoted as 'non-bleached litter'. Hyphal length, water content, chemical property, and fungal species composition were investigated for the bleached and non-bleached litter.

Hyphal length was estimated for litter samples collected on December 2000 by the method of Jones and Mollison (1948) as modified by Ono (1998). The method is described above, except that 6 agar films were prepared for each sample.

Water content and chemical analyses were measured for litter samples collected on December 1999 and December 2000. The litter samples were dried at 40°C for 7 days to determine field water content. Concentrations of lignin, holocellulose, soluble carbohydrate, and polyphenol were measured according to the methods described in Chapter 2. Concentrations of nitrogen, phosphorus, potassium, calcium, and magnesium were measured according to the methods described in Chapter 4. Nitrogen transformation rates were determined by aerobic laboratory incubation. About 5 g from each litter sample were incubate for 28 days at 30°C during which the initial water content was maintained. Additional litter samples were dried at 105°C to determine field moisture content. Both incubated and unincubated samples were extracted using 2 M KCl. NH_4-N concentrations were measured by the indophenol blue method and NO_3-N concentrations were measured colorimetrically following Zinc reduction rather than Cadmium reduction (Keeney and Nelson 1982). Net mineralization rates were calculated by subtracting initial inorganic N (NH_4-N + NO_3-N) concentrations from final concentrations. Net nitrification rate was calculated by subtracting initial NO_3-N concentration from final NO3-N concentration. Percent (%) nitrification represents the percentage of net nitrification rate to net mineralization rate.

Isolation of fungi was carried out for bleached litter collected on November 1999. A total of sixty litter fragments (approx. 5 x 5 mm), thirty bleached litter and thirty non-bleached litter, were processed with the washing method. Fungal isolation was carried out within 8 hours after sampling. The methods are described in Chapter 2, except that two disks were placed on the surface of each LCA plate. Frequency of a species is calculated as a percentage of the number of disks with the species to the total number of disks tested in each litter type (30). When comparing the frequency of a species between litter types, Fisher's exact probability test on 2 x 2 contingency tables was used, because data were in the form of proportions.

Table 3.1 Frequency (%) of fruit bodies of the Basidiomycota

Fungus	Upper	Lower	Soil horizon
Mycena filopes	29.6	60.0	LA
Mycena polygramma	13.6	15.2	L
Mycena sp.	4.0	8.0	LA
Collybia peronata	4.0	0.8	L
Collybia sp.	4.0		L
Hydnum repandum	2.4		A
Stropharia aeruginosa	1.6	0.8	L
Hygrocybe cantharellus	1.6		L
Marasmius sp.	0.8	0.8	LA
Lepiota fusciceps	0.8		F
Naematoloma sublateritium	0.8		A
Lycoperdon perlatum		12.8	LA
Mycena pura		10.4	L
Mycena amicta		4.0	L
Psathyrella candolleana		4.0	LA
Mycena crocata		2.4	L
Mycena luteopallens		2.4	L
Unidentified B2		2.4	L
Unidentified B6		2.4	L
Agaricus praeclaresquamosus		1.6	L
Clitocybe sp.		1.6	L
Pseudoclitocybe cyathiformis		1.6	L
Lepiota cf. *pseudogranulosa*		0.8	L
Lepiota cygnea		0.8	L
Lepiota sp.		0.8	A
Marasmius pulcheriipes		0.8	L
Mycena cf. *osmundicola*		0.8	L
Psathyrella piluliformis		0.8	L
Volvariella speciosa var. *gloiocephala*		0.8	L
Unidentified species	1.6	3.2	L
Number of species	13	28	
Litter decomposing fungi (total)	64.8	140.0	
Mycorrhizal fungi (total)	53.6	1.6	

Fruit bodies were collected from 500 m^2 in upper and lower sites for 9 times from May to November 2001. Frequency = number of grid (2 x 2m) with the fruit body / total number of grid (125) x 100.

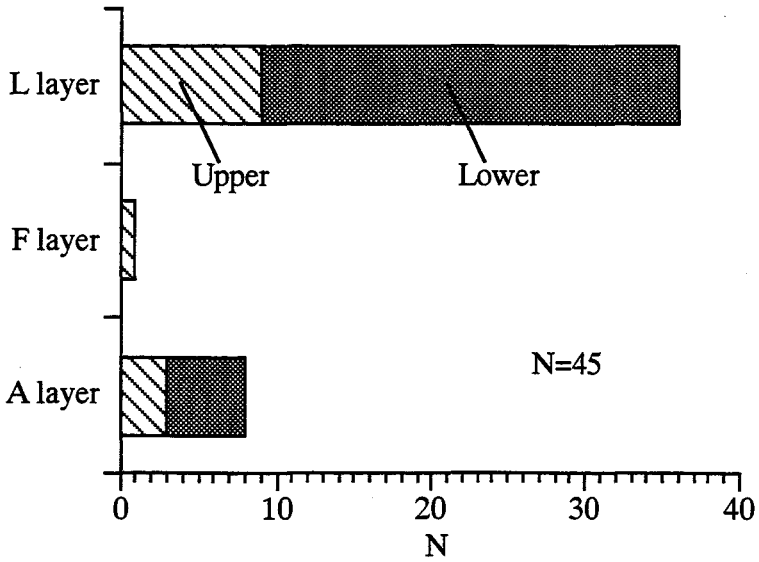

Fig. 3.1 Frequency distribution of soil horizons from which fruit body of litter decomposing fungi were emerged

Results

Fruit body observations

Table 3.1 shows frequency of fruit bodies of the Basidiomycota in upper and lower sites. In both sties, litter decomposing fungi occurred more frequently than mycorrhizal fungi. A total of 35 species was collected for litter decomposing fungi. Total frequency and number of species of litter decomposing fungi were higher in lower site than in upper site. The most frequent species of litter decomposing fungi were *Mycena filopes* and *M. polygramma* in both sites. Total frequency of mycorrhizal fungi was higher in upper site than in lower site. The occurrence of mycorrhizal fungi was very low in lower site.

Figure 3.1 shows frequency distribution of soil horizons from which fruit body of litter decomposing fungi was emerged. Of 45 cases of the fruit body occurrence, 36 cases (80%) emerged from the L layer. Only one species (*Lepiota fusciceps*) occurred from F layer in upper site.

Figure 3.2 shows seasonal pattern of occurrence of fruit body. In the upper and lower sites, phenology of fruit body of litter decomposing fungi had two peaks: the first peak during late-May to late-June and the second peak during mid-September to early November.

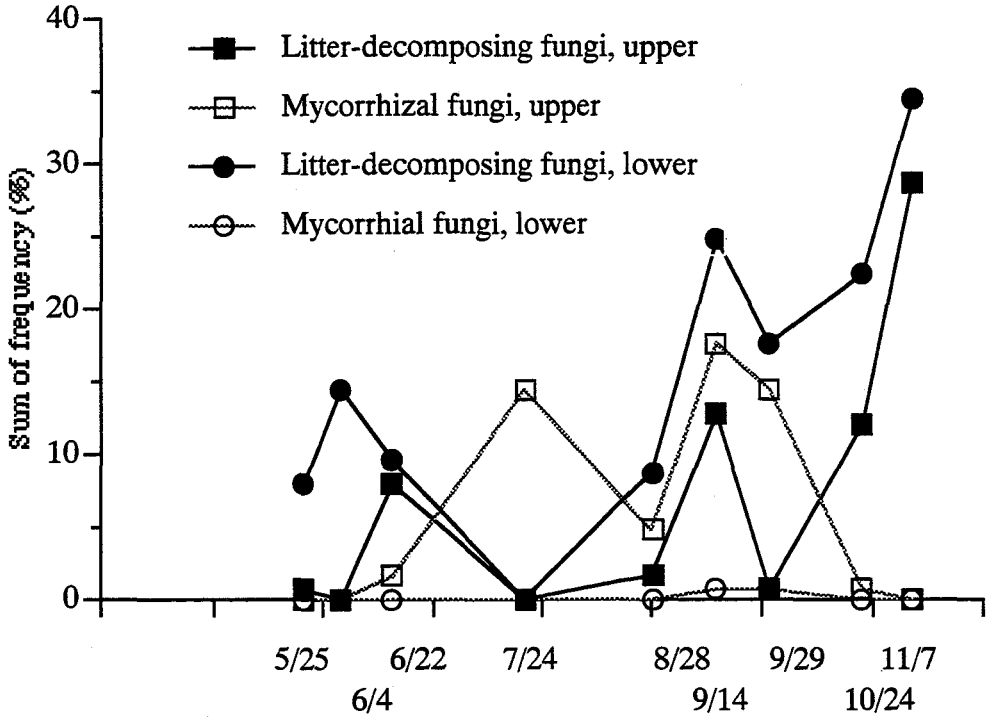

Fig. 3.2 Seasonal pattern of occurrence of fruit body

Table 3.2 Fungal biomass in soil horizons. Standard errors in parenthesis.

	Total (mg/g dry material)	Clamp-bearing (mg/g dry material)	% clamp-bearing to total	n
Upper				
L	5.9 (0.6)	1.0 (0.3)	14 (3)	25
F	4.2 (0.5)	0.5 (0.2)	9 (3)	20
A	0.9 (0.1)	0.0 (0.0)	1 (1)	18
Lower				
L	4.3 (0.3)	0.4 (0.1)	9 (2)	25
F	1.4 (0.2)	0.1 (0.1)	3 (3)	5
A	0.5 (0.1)	0.0 (0.0)	0 (0)	18

Phenology of fruit body of mycorrhizal fungi in upper site also had two peaks: the first peak on late-July and the second on mid-September.

Fungal biomass in soil horizons

Table 3.2 shows fungal biomass in soil horizons in upper and lower sites. In both sites, total fungal biomass was L > F > A layers in order. In both sites, clamp-bearing fungal biomass was L > F > A layers in order. Clamp-bearing biomass was negligible at A layer.

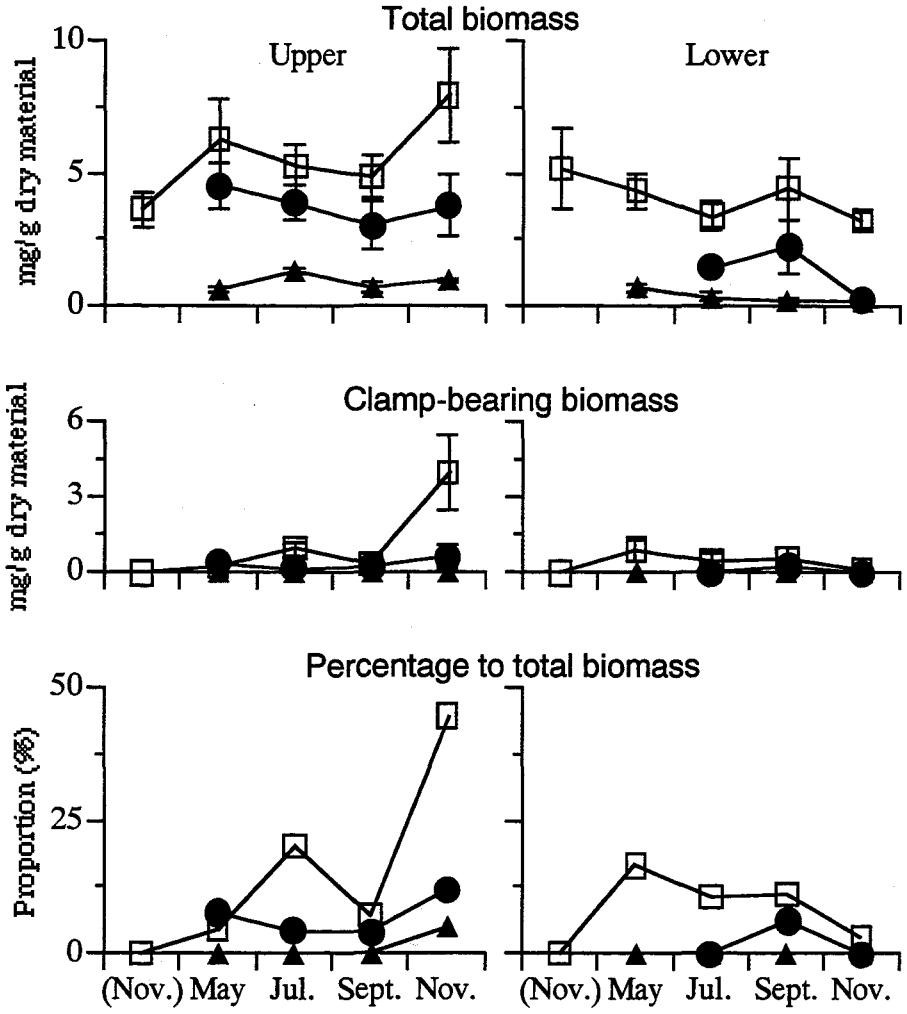

Fig. 3.3 Seasonal changes in fungal biomass at L (open box), F (black circle), and A (black triangle) layers in upper (left) and lower (right) sites. (Nov.) indicates the freshly fallen litter collected on November 2001. Bars indicate standard errors (n=3 to 5).

Clamp-bearing biomass was higher in upper site than in lower site. Proportion of clamp-bearing biomass to total biomass was L > F > A layers in order.

Figure 3.3 shows seasonal changes in fungal biomass in L, F, and A layers. In upper site at L layer on November, total fungal biomass, clamp-bearing fungal biomass, and percentage of clamp-bearing biomass to total biomass were significantly (ANOVA, $P<0.05$, Scheffe test) lower in freshly fallen litter than in partly decomposed litter. In upper site, total fungal biomass at A layer also varied seasonally. Total biomass on May was significantly (ANOVA, $P<0.05$, Scheffe test) lower than that on June. In upper site, clamp-bearing fungal

Fig. 3.4 Bleached litter associated with fruit body of *Clitocybe* sp. (September 2001)

biomass and its percentage to total biomass at A layer varied seasonally and were significantly (ANOVA, $P<0.05$, Scheffe test) higher on November than those on the other months. No significant seasonal trend was found in total fungal biomass, clamp-bearing fungal biomass, and its percentage to total biomass at F layer in upper site and at L and A layers in lower site. The ANOVA was not applied to F layer in lower site because of low sample number.

Bleached litter

In the study site, litter materials that suffered bleaching by the Basidiomycota were observed during September to December. The bleached litter occurred sparsely on forest floor, but occasionally encountered on lower to bottom part of forest slopes. The bleached litter was commonly found on thin layer between L layer and mineral soil. In most cases, the bleached litter was associated with fruit body of the Basidiomycota, mostly *Clitocybe* sp. and less frequently *Collybia peronata* and *Mycena polygramma*. An example of bleached litter

Table 3.3 Hyphal length, water content, and chemical property of bleached litter produced by *Clitocybe* sp. in comparison with surrounding, non-bleached litter. Standard deviations in parenthesis. nd, not determined.

	1999 (n=3)		2000 (n=5)	
	Bleached litter	Non-bleached litter	Bleached litter	Non-bleached litter
Hyphal length (m/g dry material)	37484 (5217)	6927 (473)	nd	nd
Water content (%)	186 (18)	243 (18)	371 (60)	342 (27)
Organic chemical constituents				
Lignin	32.4 (1.2)	42.2 (0.6)	35.5 (3.8)	45.5 (1.1)
Holocellulose	22.9 (1.2)	20.2 (2.1)	16.8 (2.1)	16.3 (0.5)
Soluble carbohydrate	2.9 (0.1)	1.9 (0.1)	3.7 (0.5)	2.2 (0.1)
Polyphenol	0.7 (0.1)	0.7 (0.0)	0.9 (0.2)	0.9 (0.1)
Nutrients				
Nitrogen	2.45 (0.04)	2.21 (0.08)	2.23 (0.10)	2.01 (0.13)
Phosphorus	0.15 (0.01)	0.10 (0.00)	0.11 (0.01)	0.08 (0.00)
Potassium	0.10 (0.01)	0.08 (0.02)	0.26 (0.03)	0.19 (0.03)
Calcium	0.86 (0.06)	0.62 (0.17)	0.69 (0.15)	0.52 (0.11)
Magnesium	0.16 (0.00)	0.12 (0.01)	0.13 (0.04)	0.10 (0.01)

Table 3.4 Inorganic-N pool size and mineralization and nitrification rates in bleached and non-bleached humus. Standard errors in parenthesis. T-test was used for the comparison.

	Bleached litter	Non-bleached litter	Probability
NH_4-N (mg/kg)	416 (185)	109 (16)	$P<0.05$
NO_3-N (mg/kg)	8 (1)	3 (0)	$P<0.001$
Net mineralization rate (mg N/kg/30d)	4655 (2065)	1128 (273)	$P<0.05$
Net nitrification rate (mg N/kg/30d)	14 (7)	5 (2)	$P=0.05$

produced by *Clitocybe* sp. is shown in Fig. 3.4.

Table 3.3 shows hyphal length, water content, and chemical property of bleached litter produced by *Clitocybe* sp. in comparison with surrounding, non-bleached litter. Hyphal length was about 5 time higher at the bleached litter than at the non-bleached litter. Water content was lower at the bleached litter than at the non-bleached litter in 2000 but was similar in 2001. Lignin concentration was lower and concentrations of holocellulose and soluble carbohydrate were higher at the bleached litter than at the non-bleached litter. Polyphenol concentration was similar at both litter types. Concentration of nutrients (nitrogen, phosphorus, potassium, calcium, and magnesium) was higher at the bleached litter than at the non-bleached litter.

Table 3.4 shows inorganic-N pool size and mineralization and nitrification rates in bleached and non-bleached litter. The NH_4-N and NO_3-N pool sizes were significantly higher at the bleached litter than at the non-bleached litter. The net mineralization rate was four times higher at the bleached litter than at the non-bleached litter and the difference was significant. The net nitrification rate was also higher at the bleached litter than at the non-bleached litter.

Table 3.5 shows frequency of microfungi on bleached and non-bleached litter. A total of 114 isolates in 34 species was isolated from the bleached litter, while a total of 207 isolates in 43 species was isolated from the non-bleached litter. *Trichoderma hamatum* and *Penicillium citrinum* were frequent on both litters. Frequencies of *T. koningii*, *Chaetomium globosum*, *P. miczynskii*, and *Geniculosporium serpens* were significantly higher at the bleached litter than at the non-bleached litter. Frequencies of *Trichoderma* sp.1, *Mucor*

hiemalis, *Mortierella verticillata*, *T. viride*, *G. roseum*, *M. ramanniana* var. *ramanniana*, *Absidia glauca*, *Cladosporium cladosporioides*, *P. velutinum*, *Mortierella* cf. *zicae*, and *M. wuyshanensis* were significantly lower at the bleached litter than at the non-bleached litter. *Clitocybe* sp. was isolated from the bleached litter with the frequency of 3.3%.

Table 3.5 Frequency (%) of microfungi on bleached and non-bleached litter.

Fungus	Bleached litter	Non-bleached litter	Probability	
Trichoderma hamatum	50.0	53.3	0.20	
Trichoderma koningii	53.3	33.3	0.06	*
Chaetomium globosum	23.3	0.0	0.01	***
Penicillium miczynskii	20.0	3.3	0.05	**
Geniculosporium serpens	13.3	0.0	0.06	*
Trichoderma sp.1	20.0	66.7	0.00	****
Mucor hiemalis	0.0	56.7	0.00	****
Mortierella verticillata	0.0	46.7	0.00	****
Trichoderma viride	30.0	46.7	0.09	*
Gliocladium roseum	10.0	46.7	0.00	***
Mortierella ramanniana var. *ramanniana*	16.7	43.3	0.02	**
Absidia glauca	0.0	33.3	0.00	****
Cladosporium cladosporioides	0.0	26.7	0.00	***
Penicillium velutinum	0.0	20.0	0.01	**
Mortierella cf. *zicae*	0.0	16.7	0.03	**
Mortierella wuyshanensis	0.0	13.3	0.06	*
Penicillium citrinum	13.3	16.7	0.26	
Mucor racemosus	13.3	6.7	0.24	
Gliocladium virens	10.0	13.3	0.29	
Gliomastix felina	10.0	3.3	0.25	
Mortierella isabellina	6.7	20.0	0.10	
Penicillium sclerotiorum	6.7	10.0	0.32	
Trichoderma pseudokoningii	6.7	10.0	0.32	
Calcarisporium arbuscula	6.7	6.7	0.39	
Trichoderma harzianum	6.7	6.7	0.39	
Verticillium cf. *suchlasporium*	6.7	6.7	0.39	
Mortierella sp.1	6.7	3.3	0.38	
Pestalotiopsis sp.1	6.7	0.0	0.25	

Paecilomyces carneus	3.3	6.7	0.38
Acremonium sp.	3.3	3.3	0.51
Arthrinium state of *Apiospora montagnei*	3.3	3.3	0.51

Table 3.5 Continued.

Fungus	Bleached litter	Non-bleached litter	Probability
Mucor piriformis	3.3	3.3	0.51
Phoma sp.	3.3	3.3	0.51
Verticillium psalliotae	3.3	3.3	0.51
Acremonium sp.	3.3	0.0	0.50
Arthrinium phaeospermum	3.3	0.0	0.50
Clitocybe sp.	3.3	0.0	0.50
Mortierella globurifera	0.0	10.0	0.12
Penicillium waksmanii	0.0	10.0	0.12
Penicillium thomii	0.0	6.7	0.25
Mortierella ramanniana var. *angulispora*	0.0	3.3	0.50
Penicillium chrysogenum	0.0	3.3	0.50
Penicillium glabrum	0.0	3.3	0.50
Penicillium janthinellum	0.0	3.3	0.50
Penicillium verrucosum	0.0	3.3	0.50
Scedosporium sp.	0.0	3.3	0.50
Trichoderma polysporum	0.0	3.3	0.50
Hyphomycete SH2-3-7	0.0	3.3	0.50
Zygorrhynchus heterogamus	0.0	3.3	0.50
White sterile mycelia	13.3	0.0	
Number of species	34	43	

The result of Fisher's exact probability test is shown. * $P<0.10$, ** $P<0.05$, *** $P<0.01$, **** $P<0.001$.

Discussion

Clitocybe, *Collybia*, *Marasmius*, and *Mycena* are the most representative genera of litter-decomposing Basidiomycota in forest soils (Hering 1982; Cooke and Rayner 1984). In this study site, *Mycena* spp. and *Collybia* spp. were frequent in terms of the number of fruit bodies and *Clitocybe* sp. was associated with the bleaching of litter. The proportion of clamp-bearing biomass to total fungal biomass in soil horizons was 0% to 14% that was within the range of previous reports (Ruscoe 1971b; Bååth and Söderström 1977; Nelson and Visser 1978; Frankland 1982; Kjøller and Struwe 1982). Most of the fruit bodies of litter-decomposing Basidiomycota emerged from L layer and clamp-bearing biomass was abundant on L layer, indicating that these fungi were active colonizers of L layer materials. The bleached litter also occurred frequently on lower part of L layer. These results are contrast with some previous studies that reported the biomass was highest at F layer (Saito 1956; Frankland 1982). In upper site, one-third of the biomass of the Basidiomycota distributed at F layer but few fruit bodies of litter-decomposers emerged from this layer. This result suggested that the mycelia within F layer might belong to mycorrhizal species. However, their mycelia are difficult to be separated from those of litter-decomposers.

There have been a few reports that compared species composition of litter-decomposing Basidiomycota between different sites. The result of the present study contrasts with Hering (1967) that reported the number and production and fruit body was lower at mull site than at moder site in England, but is consistent with Rastin et al. (1990) that reported the production of fruit body was higher at lower slope than at upper slope in a spruce forest in Germany. Seasonal patterns of the Basidiomycota was also reported. Hering (1967) reported the seasonal peak of fruiting. Ruscoe (1971b) found the length of basidiomycete hyphae in H layer increased in Autumn.

There has been no data on the mycelial abundance of the Basidiomycota between different sites. In the present study, clamp-bearing biomass was higher in upper site than in

lower site. However, this did not necessarily indicate the activity of litter-decomposing Basidiomycota was higher in upper site than in lower site. Clamp-bearing biomass in upper site increased rapidly on November, but this increase is difficult to be ascribed to mycelia of litter-decomposing species too, because whether these mycelia belonged to litter-decomposing fungi or mycorrhizal fungi was unclear.

Therefore, these results indicated that the activity of litter-decomposing Basidiomycota was highest at L layer. However, the site difference and seasonal change of their activity were difficult to evaluate. This is because occurrence of fruit bodies is a poor guide to the activity and because the mycelia of litter-decomposing species are impossible to be separated from those of mycorrhizal species unless the production of antibody specific to a litter-decomposing species (Frankland et al. 1981; Hitchcock et al. 1997).

On the other hand, bleaching of forest litter represents litter-decomposing activity of the Basidiomycota. The occurrence of bleached litter around their fruit bodies has already reported by Harris (1945), Saito (1966), and Hintikka (1970). In the study site, the bleached litter was mostly associated with *Clitocybe* sp. and less frequently with *Collybia peronata* and *Mycena polygramma*. In Japan, *Marasmius* species such as *M. purrcherius* and *M. maximus* also bleach leaf litter (Koide 2002, Osono personal observation). In addition, Hintikka (1970) reported species in *Clavaria*, *Cudonia*, *Cystoderma*, *Lepiota*, *Psalliota*, *Rhodocybe*, and *Spathularia* as bleaching fungi in Finland. Hintikka (1970) estimated the amount (% cover) of bleached litter to be 0.4% to 11.3% of the surface of forest floor.

Lower lignin concentration and higher net N mineralization rate in bleached litter compared to non-bleached litter indicated the selective delignification was associated with N mineralization in litter. This is consistent with Osono and Takeda (2001b) that reported N mineralization from beech litter was coincided with the decomposition phase of lignin-N complex by basidiomycetous fungi. The 'lignin' fraction in partly decomposed litter contained not only true plant lignin but also secondary lignin-like humic substances synthesized during decomposition. The Basidiomycota are able to attack humic acids by

ligninolytic enzyme system (Blondeau 1989). Therefore, delignification by the Basidiomycota was associated with nitrogen mineralization from the litter.

A variety of fungi were isolated from bleached litter and the frequency of *Clitocybe* sp. was only 3.3%. This was due to the isolation method was selective for fast-growing species such as *Trichoderma* spp. (Osono and Takeda 1998). This did not mean that *Clitocybe* sp. had little effect on the production of bleached litter. Rather, *Clitocybe* sp. produced the dominant effects, as the fungus brought about similar changes when inoculated to litter in pure culture while other microfungi frequent on bleached litter caused a limited decomposing ability (Chapter 6).

Species composition of microfungi on bleached litter was different from that on non-bleached litter. Most of the late occurring species of beech leaf litter, denoted as 'secondary sugar fungi' in Osono and Takeda (2001b), were also frequent on non-bleached litter but decreased their frequencies on bleached litter. Instead, *Chaetomium globosum*, *Penicillium miczynskii*, and *Geniculosporium serpens* that were rare or absent on non-bleached litter increased their frequencies on bleached litter. These change in species composition may be ascribed to the increase of the availability of readily available resources such as delignified holocellulose and soluble carbohydrates during the delignification process by *Clitocybe* sp.

According to Hintikka (1970), fungi with bleaching activity showed a preference for the thick litter layers but bleached litter seemed to be connected with mull formation in brown forest soils in deciduous forests. Hintikka (1970) concluded that the bleaching activity cannot be directly correlated with the type of humus layer. From the result of the present study, the bleaching activity was difficult to relate to the development of moder and mull soils on upper and lower parts of forest slopes. However, the observation that the bleached litter occasionally encountered on mull soil at lower to bottom part of forest slopes suggested a possible relationship between the bleaching activity of the Basidiomycota and mull soil formation. The amount of bleached litter should be compared quantitatively to evaluate the relationship.

Chapter 4

Organic chemical and nutrient dynamics in decomposing leaf litter in relation to fungal ingrowth and succession

Introduction

In forest ecosystems, decomposition of plant litter is an important factor controlling nutrient cycling and soil humus formation. The litter decomposition processes are regulated by the resource availability for decomposer organisms such as organic and nutrient chemistry of the litter and by the environmental factors that affect the activity of the decomposers such as temperature and moisture (Swift et al. 1979; Heal et al. 1997). The study of the relationship between chemical and biological changes during the litter decomposition is thus important in understanding organic matter and nutrient dynamics in soil systems (Takeda 1994).

Nitrogen and phosphorus are essential elements that limit not only plant growth but growth of microbial populations in the soil systems (Beever and Burns 1980; Jennings 1989). Nitrogen and phosphorus dynamics in decomposing litter show leaching, immobilization, and mobilization phases (Berg and Staaf 1981; Staaf and Berg 1982) and have been related to the availability of organic chemical energy sources to the decomposers (Berg 1986; Melillo et al. 1989; Aber et al. 1990) and the ingrowth of fungal populations (Berg and Söderström 1979; Hasegawa and Takeda 1996). Net release (i.e. mineralization) of nitrogen and phosphorus begins at critical carbon to nutrient ratios (Takeda 1998) or when the amount of lignin starts decreasing (Berg and McClaugherty 1989). Potassium, calcium, and magnesium are also essential nutrients necessary for plant growth. Potassium leached out quickly from decomposing litters, calcium decreased as carbon loss during litter decomposition, and magnesium often showed an intermediate release pattern (Gosz et al. 1973; Staaf and Berg 1982; Blair 1988; Hasegawa and Takeda 1996).

Lignin and holocellulose in the litter structure are major energy sources available to decomposer organisms, constituting 70-80% of fresh organic material (Swift et al. 1979). Lignin is less readily available to the decomposers than holocellulose and often retards litter decomposition (Fogel and Cromack 1977; Berg et al. 1982; Takeda et al. 1987) because: (i) lignin, an aromatic compound made up of phenylpropane-based monomers linked via a variety of bonds, is highly refractory and persistent and the delignification depends on the availability of non-lignified carbon energy sources (Kirk et al. 1976); (ii) lignin forms a resistant shield around holocellulose to form lignocellulose in plant cell walls (Cooke and Whipps 1993) and, as a consequence, most of the holocellulose in litter must be delignified for carbohydrate assimilation; and (iii) lignin decomposition products may form stable nitrogenous compounds making nitrogen less readily available to decomposer organisms (Berg 1988). Lignocellulose index (LCI), relative availability of holocellulose in lignocellulose matrix, is thus suggested as a useful index of availability of carbon energy sources to decomposer organisms (Berg et al. 1984; Melillo et al. 1989; Aber et al. 1990).

Among the decomposer organisms, fungi play an important role in litter decomposition (see General Introduction). There have been two approaches to evaluate the function of the fungal community in litter decomposition. In the first approach, decomposer microbial community is considered as 'functional black box' and total fungal biomass (Berg and Söderström 1979; Berg and Wessén 1984; Berg 1991; Osono et al. submitted) or total enzymatic activity (Sinsabaugh et al. 1991; Sinsabaugh 1994) are estimated and related to organic chemical and nutrient dynamics during litter decomposition, component fungal species being left out of consideration. In the second approach, fungal species composition on litters is investigated first and the functional role of each species is then evaluated by substrate utilization tests (Kjøller and Struwe 1980, 1987, 1990; Rosenbrock et al. 1995; Dilly et al. 2001) or by *in vitro* decomposition tests (Lindeberg 1946; Saito 1960; Hering 1967; Kuyper and Bokeloh 1994; Osono and Takeda 1999b; Miyamoto et al. 2000; Osono and Takeda 2002a). A few studies has been, however, carried out to relate the function of component

fungal species to organic chemical and nutrient dynamics during litter decomposition.

Fungal succession during litter decomposition has been observed on several litters of forest tree species (Hudson 1968; Swift 1976). In earlier works, successional changes in fungal populations were investigated by dilution plating method (Saito 1956; Ishii 1968; Deka and Mishra 1982; Kuter 1986; Singh *et al.* 1990). Most fungi isolated by this method are, however, derived from dormant spores attached to litter surface (Warcup 1955; Christensen 1969). Hence the relationship between litter decomposition processes and fungal succession described was unclear. To detect the function of fungal species in the decomposition processes, alternatively, a surface sterilization method that isolates fungi present within internal tissues (Kinkel and Andrews 1988; Hata 1997) and a washing method that removes propagules on the surface and isolates actively growing mycelia (Harley and Waid 1955; Tokumasu 1980) have been developed and assured of their usefulness on several litter types (Kendrick and Burges 1962; Macauley and Thrower 1966; Tokumasu 1996; Osono and Takeda 1999b, 2001a).

Based on these methods, fungal succession has been investigated during litter decomposition. Recent studies using the litter bag method have reported that colonization of fungal populations was related to particular stages in decomposition (Hering 1965; Slapokas and Granhall 1991; Attili and Tauk-Tornisielo 1994; Robinson et al. 1994; Pasqualetti et al. 1999). More detailed studies are thus required to evaluate the effect of chemical changes on fungal succession on decomposing litter. Furthermore, most of the previous studies covered less than a one year period and few studies have been carried out during long term decomposition processes. Beech leaves follows decomposition pattern typical of temperate tree species and its slowly decomposing leaves are suitable for a long term study (see General Introduction).

The purposes of this study are to evaluate: (i) the function of fungal populations in the organic chemical and nutrient dynamics; and (ii) the effect of resource quality on the fungal succession on decomposing beech leaf litter. Litter bag experiments were thus

performed to follow the changes in carbon, nutrient (N, P, K, Ca, Mg) and organic chemical constituents (lignin, holocellulose, soluble carbohydrate, polyphenol) in the litter. Concurrently, the changes in total and live fungal biomass, clamp-bearing fungal biomass (biomass of basidiomycetous fungi) and the fungal succession were investigated and related to the changes in litter chemistry.

Materials and methods

Study area

The study was carried out in the Ashiu Experimental Forest of Kyoto University (see Material and Study Site). Two study sites were chosen that were located on a northwest facing slope about 200 m long. One site was located on the upper part of the slope and the other on the lower part (Chapter 1). A study plot of 20 x 10 m in area was laid out in each site and was divided into 10 subplots of 4 x 5 m for the survey of litter weight and chemical changes. An additional 3 subplots of 4 x 5 m were laid out within each plot for fungal investigation.

Litter bag method

Decomposition processes of beech leaf litter were studied by a litter bag method (Crossley and Hoglund 1962). Freshly fallen leaves of beech were collected from forest floors in the study area during November 1996, the peak period of litter fall (Takeda and Kaneko 1988). The leaves were taken to the laboratory and oven-dried at 40°C for one week. The litter (3 g) was enclosed in a litter bag (15 x 15 cm) made of polypropylene shade cloth with a mesh size of approximately 2 mm. A total of 260 bags was prepared. Initial samples (approx. 10 g) were preserved for chemical analyses.

The decomposition study covered over a 35 month period from December 1996 to November 1999. Litter bags were placed on the litter layer, 10 sets in each of 26 subplots, on December 1996. The litter bags were attached to the forest floor by metal pins to prevent movement and to ensure a good contact between the bags and the litter layer. Sampling of the bags took place 10 times, at 5 (May 1997), 7 (July 1997), 9 (September 1997), 11 (November 1997), 16 (April 1998), 19 (June 1998), 21 (September 1998), 23 (November 1998), 29 (May 1999), and 35 month (November 1999) after the placement. On each sampling occasion, 26 bags were collected from 26 subplots, placed in paper bags, and taken to the laboratory.

Foreign plant remains attached to the outside the bags were carefully removed with

forceps. The losses of dry weight were determined after drying the samples in 20 bags to a constant weight at 40°C and mean values of weight loss were calculated for each sampling. The samples were then combined, ground in a laboratory mill to pass a 0.5 mm screen, and used for chemical analyses as described below. The other six bags were used for fungal biomass estimation and fungal isolation.

Decomposition rate of the litter was calculated by Olson's k (Olson 1963) according to the following equation:

$$Wt = W_0 \times \exp(-kt)$$

where Wt is the litter weight after a given period, W_0 is the original litter weight, k is the decomposition rate, and t is the year.

Chemical analyses

The amount of lignin in the samples was estimated by gravimetry according to a standardized method using hot sulfuric acid digestion (King and Heath 1967). Total carbohydrate content was estimated by the phenol-sulfuric acid method (Dubois et al. 1956) according to the method described in Fukui (1969). Soluble carbohydrate and polyphenol were extracted from the sample with 50% methanol (v/v) at 75°C for 60 min. Soluble carbohydrate content was estimated by the phenol-sulfuric acid method. Polyphenol content was estimated by the Folin-Ciocalteau method (Waterman and Mole 1994). The methods are described in Chapter 2.

Total carbon and total nitrogen contents were measured by automatic gas chromatography (NC analyzer SUMIGRAPH NC-900, Sumitomo Chemical Co., Osaka, Japan). After an acid wet oxidation in HNO_3 + $HClO_4$, the following analyses were performed; molybdate-ascorbic acid method for phosphorus (Olsen and Sommers 1982), flame photometry for potassium and atomic absorption for calcium and magnesium (Atomic

absorption spectrophotometer 170-30S, Hitachi Ltd., Tokyo, Japan).

Carbon to nutrient (N, P, K, Ca, Mg) ratios and lignin to nutrient ratios are useful indices of the litter chemical quality (Takeda 1998) and are calculated according to the following equations:

C/nutrient = carbon conc. (%) / nutrient conc. (%)

L/nutrient = lignin conc. (%) / nutrient conc. (%)

Lignocellulose index (LCI) is a useful index of availability of carbon energy sources to decomposer organisms (Berg et al. 1984; Mellilo et al. 1989; Aber et al. 1990) and is calculated according to the following equations:

LCI = holocellulose conc. (%) / (lignin conc. (%) + holocellulose conc. (%))

Fungal biomass estimation

Fungal biomass was measured by the method of Jones and Mollison (1948) as modified by Ono (1998). The method is described in Chapter 3, except that litter (1 g, fresh weight) from each of 6 litter bags were used; Six agar films were prepared for each sample. Mean diameters were 2.0 and 2.1 μm for the total hyphae, 1.8 and 1.7 μm for the live hyphae and 3.1 and 2.8 μm for the clamp-bearing hyphae in the upper and the lower site, respectively.

Fungal isolation

A surface sterilization method (Kinkel and Andrews 1988; Hata 1997) and a modified washing method (Harley and Waid 1955; Tokumasu 1980) were used for isolation of fungi. The surface sterilization was carried out only for beech leaves in litter bags collected at November 1997 (11th month). Fungal isolation was carried out within 8 hours after sampling. At each sampling occasion, 60 leaf disks were punched out with a sterile cork borer

(5.5 mm in diameter) from the central part of the leaves in 6 bags. The methods are described in Chapter 2, except that two disks were placed on the surface of each plate.

Frequency of a species is calculated as a percentage of the number of disks with the species to the total number of disks tested in each site at each sampling occasion. Species with frequency more than 20% in each sample are arbitrarily regarded as frequent.

Statistical analyses

Analysis of variance (Systat 1992) was used to determine differences between mean values of total, live, and clamp-bearing fungal biomass of 10 sampling occasions during decomposition. The Tukey's Honestly significant difference (HSD) test was used for multiple comparisons. Colonization patterns of fungal species during decomposition were classified using cluster analysis. Cluster analysis results in a hierarchical dendrogram showing species-linkages in a criterion similarity (Pearson's correlation coefficient). In this study, the group average method was used.

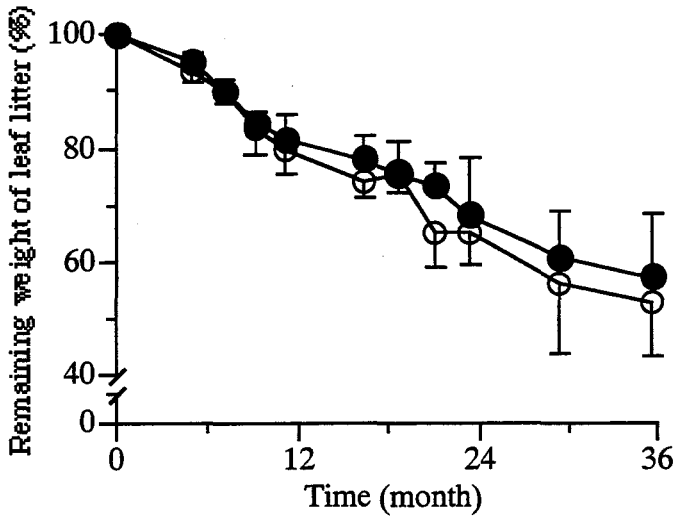

Fig. 4.1 Changes in remaining weight of beech leaf litter during decomposition. Bars indicate standard deviations (n=10). ○ = upper, ● = lower.

Results

Carbon and nutrient dynamics

Figure 4.1 shows changes in remaining weight of leaf litter during decomposition. About 53% and 57% of the original litter weight remained at the end of the study period. The decomposition rates (Olson's k) over a 35 month period were 0.222 and 0.193 in the upper and the lower site, respectively.

Figure 4.2 shows changes in remaining weight and concentration of carbon and nutrients during decomposition. Weight changes are presented as the percentage to the initial weight. Changes in carbon weight were similar to those in the litter weight. Carbon concentration decreased during decomposition. Carbon dynamics were similar between the sites.

Nutrients were categorized into two types according to the dynamics in the decomposing litter. The first type includes nitrogen and phosphorus. Weight changes in nitrogen and phosphorus were characterized by two phases: (i) immobilization in the first 21 months; and (ii) mobilization from the 21st to the 35th month. The initial net immobilization phase was characterized by an absolute increase of nitrogen and phosphorus weights due to

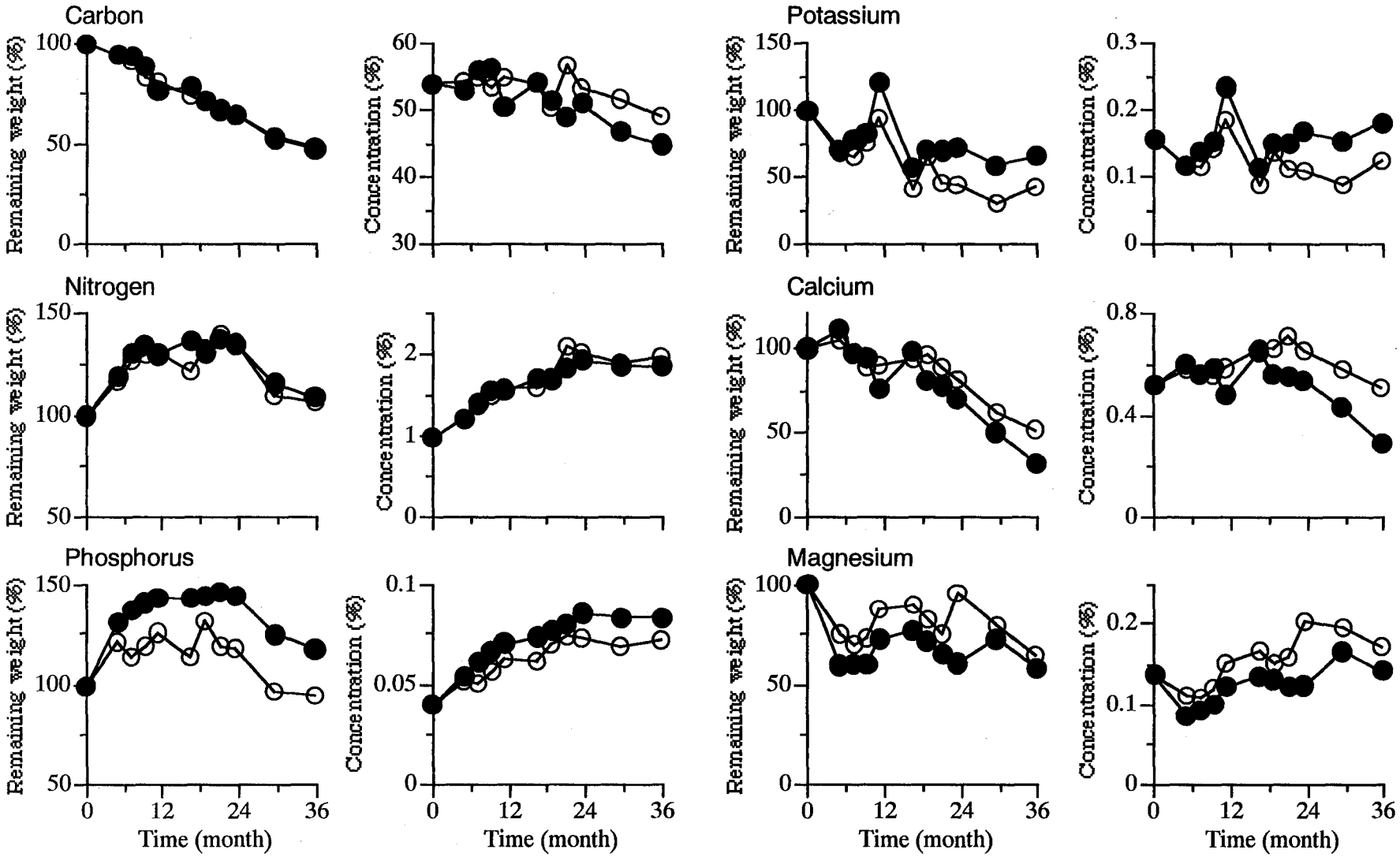

Fig. 4.2 Changes in remaining weight and concentration of carbon and nutrients during decomposition. ○ = upper, ● = lower.

the incorporation into the litter from the surrounding. Nitrogen and phosphorus concentrations increased during the immobilization phase and were constant during the mobilization phase in both sites. The second type includes potassium, calcium, and magnesium. Weight and concentration of potassium were variable throughout the study period in both sites. Calcium weight increased in the first 5 month and then decreased thereafter in both sites. Calcium concentration increased in the first 16 month and 21 month in the upper and the lower site, respectively, and then decreased thereafter. Magnesium weight decreased in the first 5 and 7 month in the upper and the lower site, respectively, and was then variable thereafter. Magnesium concentration decreased in the first 5-7 month and then increased thereafter. The rate of loss of these nutrients after 35 months was N < P < Mg < Ca < K in the upper site and P < N < K < Mg < Ca in the lower site in order. Dynamics of these nutrients were similar between the sites except that remaining weight and concentration of phosphorus were higher in the lower site than in the upper site.

Carbon to nutrient ratio and lignin to nutrient ratio

Figure 4.3 shows changes in carbon to nutrient ratios during decomposition. C/N was initially 55.2 and decreased to 26.9 and 26.6 during the immobilization phase in the upper and the lower site, respectively. The decreases were then slowed down during the mobilization phase to approach asymptotes. The final C/N was 24.9 and 24.1 in the upper and the lower site, respectively. C/P was initially 1335 and decreased to 764 and 610 during the immobilization phase in the upper and the lower site, respectively. The decreases were then slowed down during the mobilization phase to approach asymptotes. The final C/P was 679 and 540 in the upper and the lower site, respectively. C/K was variable throughout the study period in both sites. C/Ca was relatively constant during decomposition in both sites. C/Mg increased in the first 5-7 months and then decreased in both sites. The changes in carbon to nutrient ratios were similar between the sites except that C/P was lower in the lower site than in the upper site.

Figure 4.4 shows changes in lignin to nutrient ratios during decomposition. L/N was

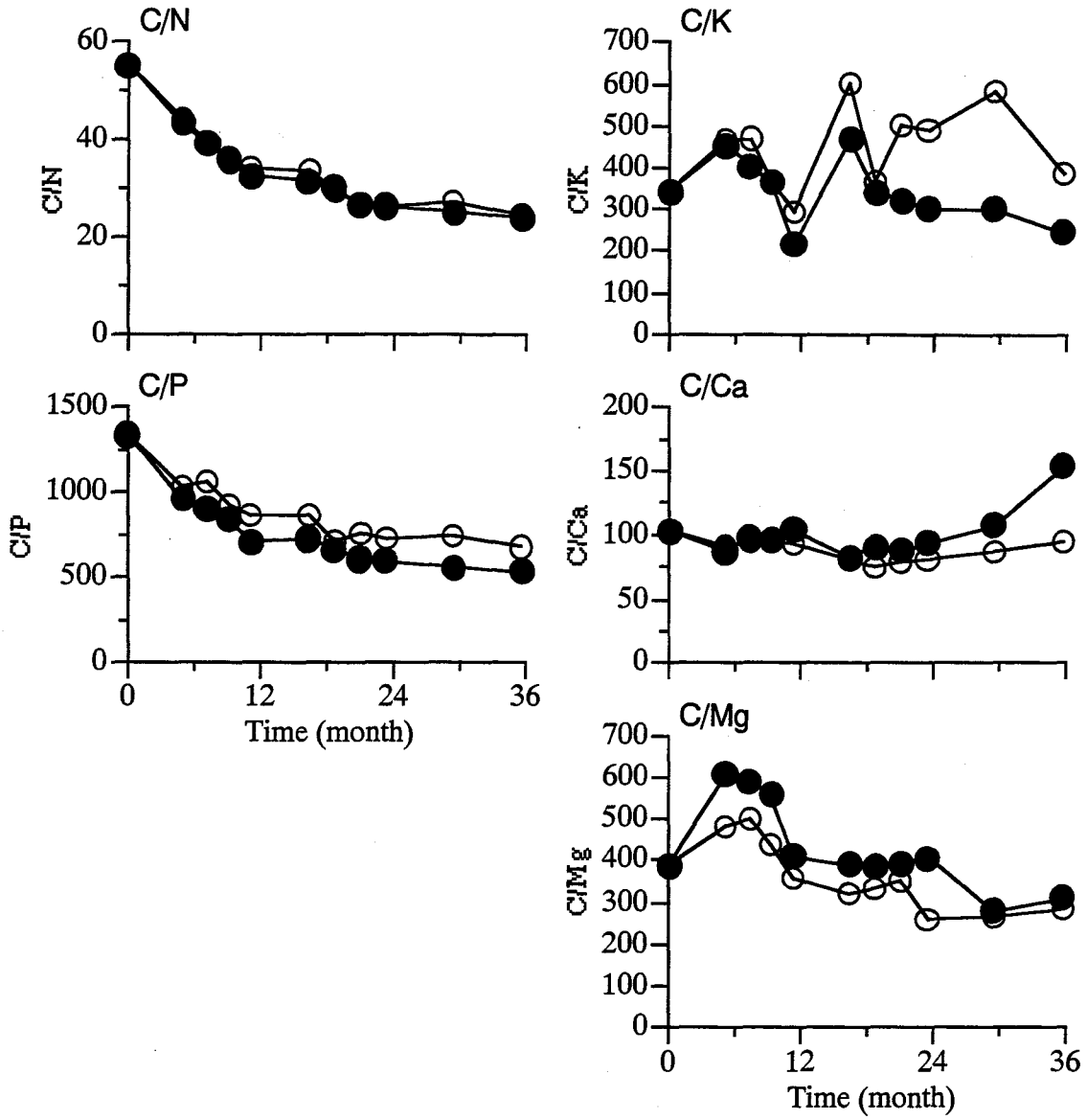

Fig. 4.3 Changes in carbon to nutrient ratios during decomposition.

○ = upper, ● = lower.

initially 44.7 and decreased to 25.9 and 27.9 during the immobilization phase in the upper and the lower site, respectively. The decreases were then slowed down during the mobilization phase to approach asymptotes. The final L/N was 25.9 and 26.3 in the upper and the lower site, respectively. L/P was initially 1080 and decreased to 736 and 640 during the immobilization phase in the upper and the lower site, respectively. The decreases were then slowed down during the mobilization phase to approach asymptotes. The final L/P was 703 and 589 in the upper and the lower site, respectively. L/K was variable throughout the study period in both sites. L/Ca was gradually increased during decomposition in both sites. L/Mg increased in the

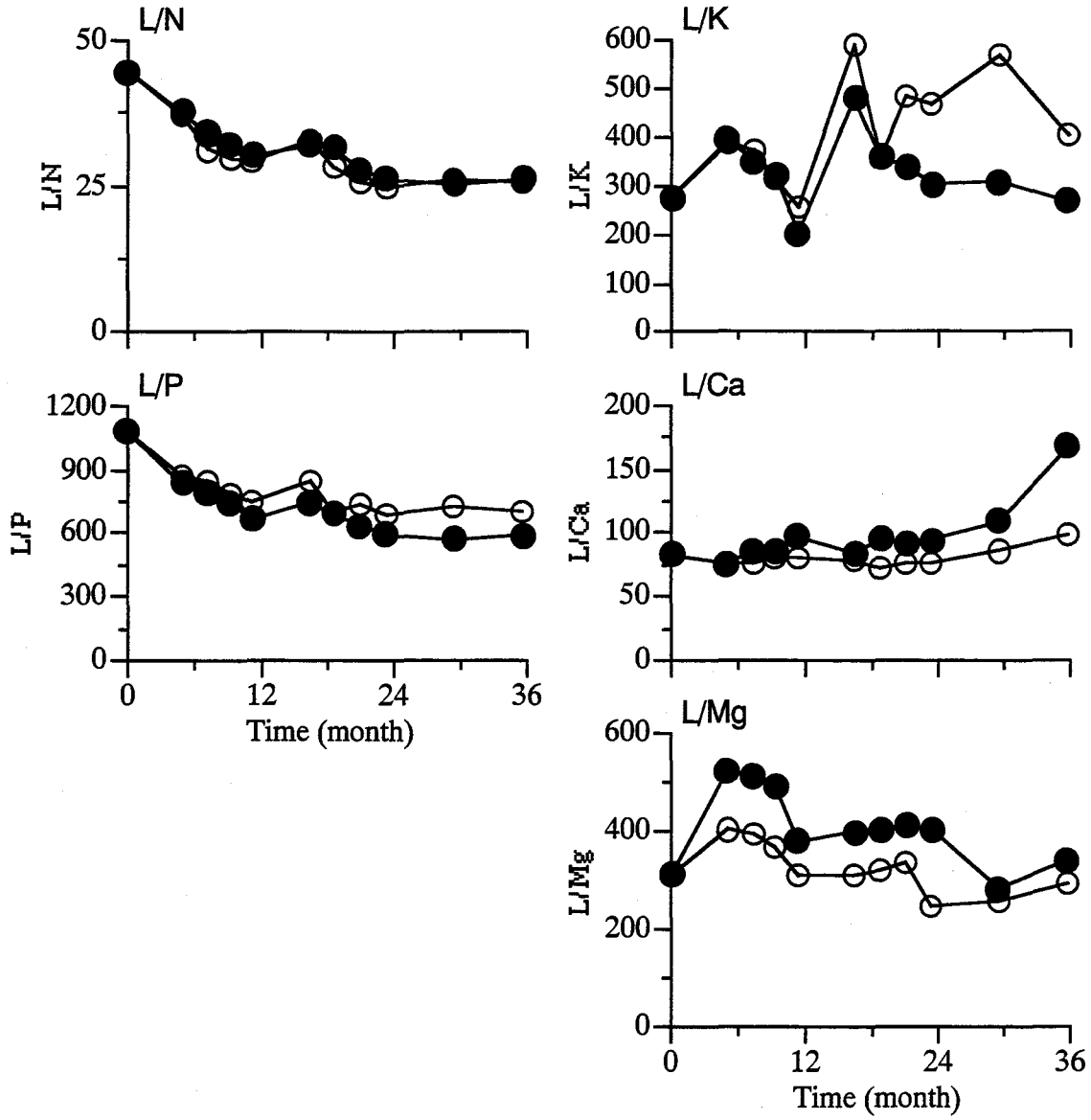

Fig. 4.4 Changes in lignin to nutrient ratios during decomposition.

○ = upper, ● = lower.

first 5 month and then decreased in both sites. The changes in lignin to nutrient ratios were similar between the sites.

Organic chemical changes

Figure 4.5 shows changes in remaining weight and concentration of organic chemical constituents during decomposition. At the end of the study period, 62% and 64% of the original lignin weight, 36% and 39% of the original holocellulose weight, 20% and 19% of the original soluble carbohydrate weight, and 17% and 14% of the original polyphenol weight

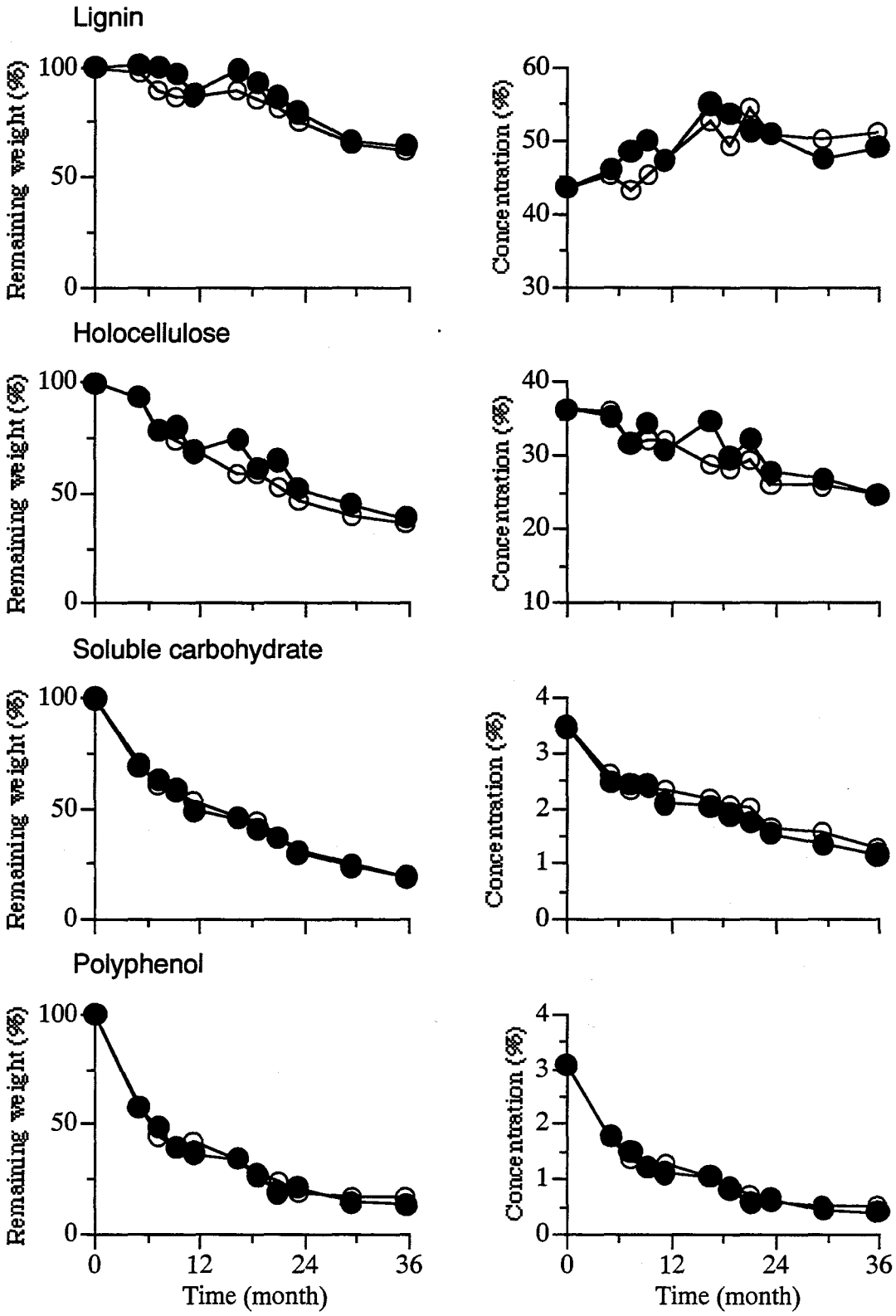

Fig. 4.5 Changes in remaining weight and concentration of organic constituents during decomposition. ○ = upper, ● = lower.

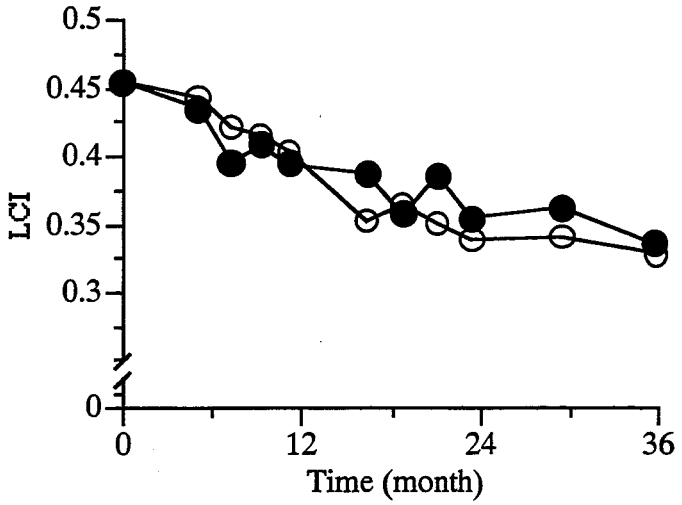

Fig. 4.6 Changes in lignocellulose index (LCI) during decomposition. ○ = upper, ● = lower.

remained in the upper and the lower site, respectively. The rate of loss of these constituents after 35 months was lignin < holocellulose < soluble carbohydrate < polyphenol in both sites in order. Lignin concentrations increased during the immobilization phase of nitrogen and phosphorus and were relatively constant during the mobilization phase. Holocellulose concentrations decreased during the immobilization phase and were relatively constant during the mobilization phase. Concentrations of soluble carbohydrate and polyphenol decreased quickly during the first 5 months and decreased constantly thereafter. Dynamics of these organic chemical constituents were similar between the sites.

Figure 4.6 shows changes in LCI during decomposition. LCI was initially 0.45 and decreased to 0.34 and 0.35 in the first 24 month in the upper and the lower site, respectively, due to the fast decomposition of holocellulose compared to lignin. The decrease was then slowed down to reach asymptotes during the mobilization phase when the disappearance of holocellulose and lignin proceeded at a similar rate. The final LCI was 0.33 and 0.34 in the upper and the lower site, respectively. The changes in LCI were significantly correlated to the changes in nitrogen and phosphorus concentrations during decomposition (Fig. 4.7). The changes in LCI were thus characterized by two phases that corresponded to the immobilization phase and the mobilization phase of nitrogen and phosphorus.

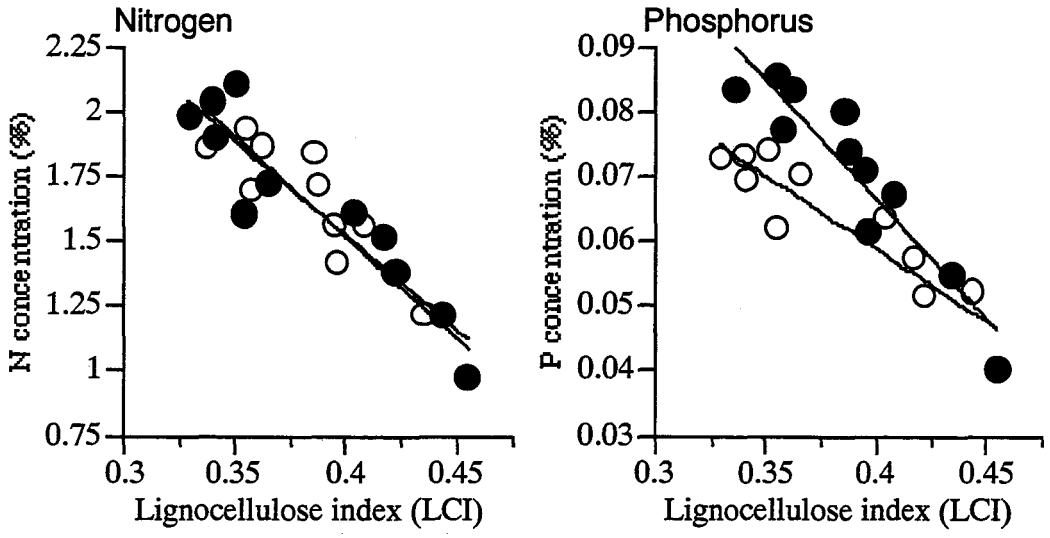

Fig. 4.7 Nitrogen and phosphorus concentrations as a function of lignocellulose index (LCI) during decomposition. ○ = upper, ● = lower. Nitrogen: upper, r^2=0.860, n=11, P<0.001; lower, r^2=0.836, n=11, p<0.001. Phosphorus: upper, r^2=0.837, n811, P<0.001; lower, r^2=0.888, n=11, P<0.001.

Changes in total, live and clamp-bearing fungal biomass

Table 4.1 shows changes in total, live, and clamp-bearing fungal biomass during decomposition. During the first year from the 5th month to the 11th month, total fungal biomass increased significantly (p<0.05, ANOVA) in both sites. The increase was positively correlated to the increase in nitrogen and phosphorus concentrations (r=0.987 and r=0.874 for nitrogen and r=0.842 and r=0.761 for phosphorus in the upper and the lower site, respectively, n=4). During the same period, live fungal biomass increased significantly (p<0.05, ANOVA) in the lower site but the difference was not significant in the upper site. Total fungal biomass and live fungal biomass then decreased and fluctuated over the rest of the study period. The mean percentage of the live biomass to the total biomass was 5.0% and 4.0% in the upper and the lower site, respectively. Dynamics of total fungal biomass and live fungal biomass were similar between the sites.

Analysis of variance indicates no significant changes in clamp-bearing fungal biomass (biomass of the Basidiomycota) during decomposition, but its percentage to total biomass increased as the decomposition proceeded, with rapid increases at the 11th month and

Table 4.1 Changes in total fungal biomass, live fungal biomass, percentage of living biomass to total biomass, clamp-bearing fungal biomass, and percentage of clamp-bearing biomass to total biomass in beech leaf litter during decomposition.

Time (month)	Total fungal biomass	Live fungal biomass	% living	Clamp-bearing fungal biomass	% clamp
Upper					
5.0	5.09 (0.98) b	0.51 (0.18) abc	10.0	0.11 (0.07) a	2.2
7.2	9.21 (1.32) ab	0.35 (0.09) abc	3.8	0.87 (0.39) a	9.4
9.2	13.53 (3.45) a	0.73 (0.12) a	5.4	0.86 (0.43) a	6.3
11.2	13.73 (1.64) a	0.60 (0.09) ab	4.4	3.50 (3.39) a	25.5
16.4	7.58 (0.72) ab	0.16 (0.05) bc	2.2	0.72 (0.31) a	9.5
18.6	5.41 (0.23) b	0.18 (0.02) bc	3.3	0.82 (0.29) a	15.2
21.0	4.25 (0.86) b	0.31 (0.11) abc	7.4	0.49 (0.37) a	11.6
23.3	8.80 (2.20) ab	0.55 (0.06) abc	6.2	1.38 (0.62) a	15.7
29.4	3.67 (0.16) b	0.12 (0.05) c	3.3	0.61 (0.32) a	16.6
35.7	6.26 (0.28) ab	0.27 (0.08) abc	4.2	1.36 (0.47) a	21.7
Lower					
5.0	4.48 (0.28) d	0.22 (0.04) bc	4.8	0.13 (0.06) a	2.9
7.2	16.28 (1.50) a	0.52 (0.04) abc	3.2	0.67 (0.44) a	4.1
9.2	14.98 (0.77) ab	0.88 (0.15) a	5.9	0.24 (0.09) a	1.6
11.2	13.17 (1.68) abc	0.59 (0.14) ab	4.5	0.84 (0.42) a	6.4
16.4	4.61 (1.32) d	0.21 (0.03) bc	4.6	0.28 (0.27) a	6.1
18.6	6.46 (1.39) cd	0.13 (0.02) c	2.1	1.01 (0.44) a	15.6
21.0	8.23 (2.38) bcd	0.25 (0.03) bc	3.0	2.83 (2.04) a	34.5
23.3	9.00 (0.88) bcd	0.50 (0.11) abc	5.5	1.20 (0.40) a	13.3
29.4	6.01 (1.44) cd	0.11 (0.03) c	1.8	1.13 (0.45) a	18.8
35.7	7.17 (1.73) cd	0.36 (0.11) bc	5.0	1.15 (0.88) a	16.0

ANOVA was used to determine differences betweeen mean values of the biomasses in each site. Standard error within parenthesis (n=3). The same letters are not significantly different at 5% level by Tukey's HSD test.

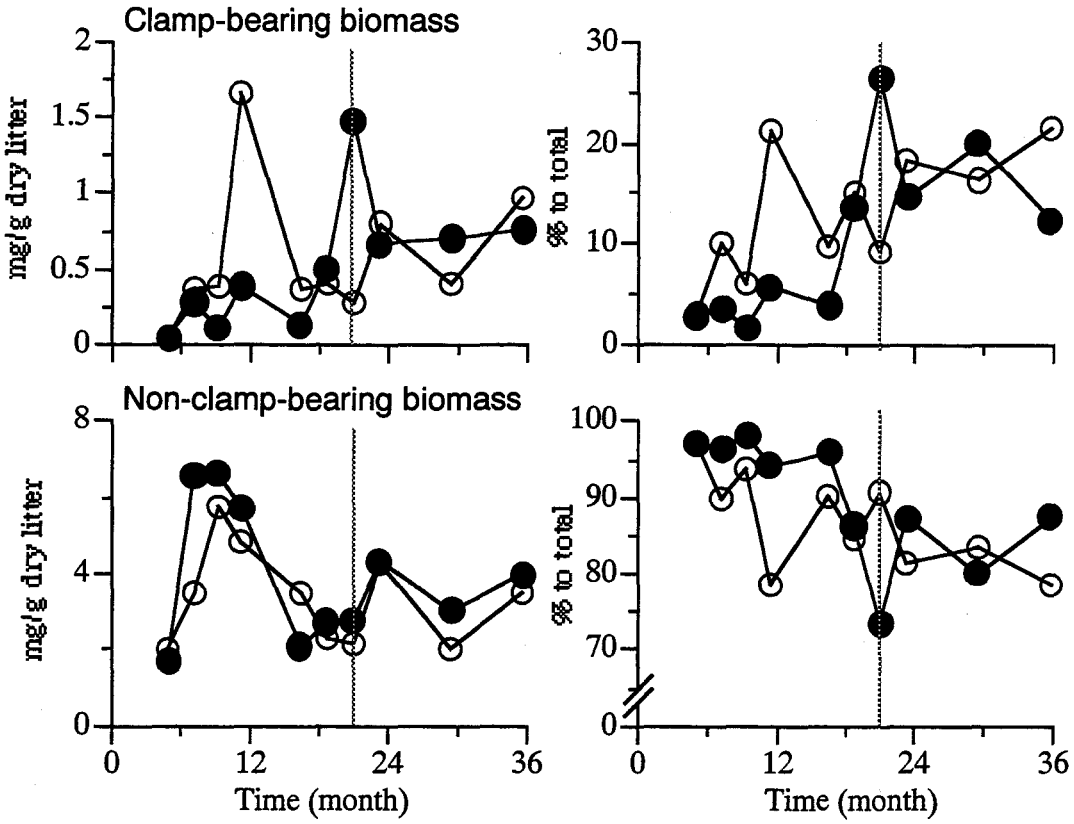

Fig. 4.8 Changes in clamp-bearing biomass and non-clamp-bearing biomass during decomposition. ○ = upper, ● = lower.

at the 21st month in the upper and the lower site, respectively (Table 4.1, Fig. 4.8). No significant relationship was found between the percentage of clamp-bearing fungal biomass and LCI. However, when the result of the rapid increases at the 11th month and at the 21st month was excluded in the analysis in the upper and the lower site, respectively, the percentage of clamp-bearing fungal biomass was significantly correlated to LCI (Fig. 4.9). The proportion of non-basidiomycetous fungi (i.e. total biomass minus clamp-bearing biomass) was initially about 100% and decreased during decomposition in both sites (Fig. 4.8).

Fungal populations

A total of 75 isolates in 14 taxa was isolated by the surface sterilization method from beech leaf litter in litter bags collected in November 1997 (11th month) (Table 4.2), including

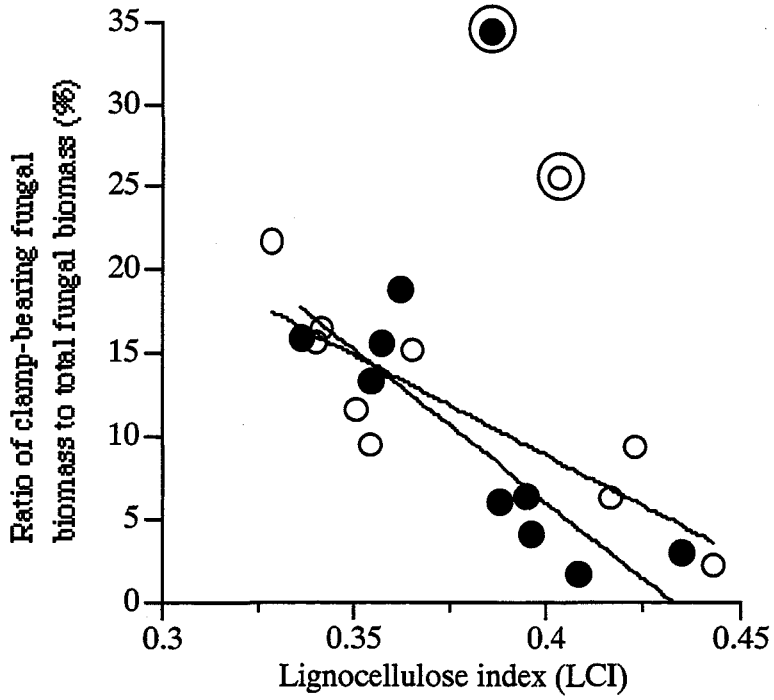

Fig. 4.9 Ratio of clamp-bearing fungal biomass to total fungal biomass as a function of lignocellulose index (LCI) during decomposition. Rapid increases in the ratio at the 11th (upper) and 21st (lower) month are circled. The lines are the regression equations with the rapid increase excluded. ○ = upper, r^2=0.746, n=9, P<0.01; ● = lower, r^2=0.785, n=9, P<0.001.

8 taxa in the Ascomycota and its anamorphs other than the Xylariaceae (denoted as the other Ascomycota), 3 in the xylariaceous Ascomycota, one in the Zygomycota, and 2 in sterile mycelia. *Geniculosporium* sp.1, an anamorphic state of *Xylaria* sp. (the xylariaceous Ascomycota) and white sterile mycelia were frequent in the upper site. *Geniculosporium* sp.1, *Xylaria* sp. (anamorph), and *Arthrinium phaeospermum* (Corda) Ellis (the other Ascomycota) were frequent in the lower site.

A total of 2027 isolates in 104 taxa was isolated by the washing method from leaf litter during decomposition (Table 4.3), including 81 taxa in the other Ascomycota, 20 in the Zygomycota, one (*Geniculosporium* sp.2) in the xylariaceous Ascomycota, and 2 in sterile mycelia. Figure 4.10 shows changes in number of species during decomposition. Number of species was variable during the immobilization phase and increased at the 35th month in both

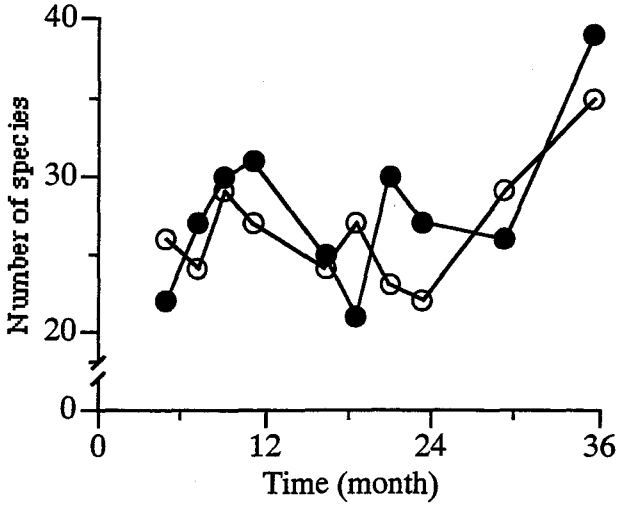

Fig. 4.10 Changes in number of species isolated by the washing method during decomposition. ○ = upper, ● = lower.

sites.

Twenty-one species in the other Ascomycota and the Zygomycota were frequent on the litter in the upper or the lower site, or in both sites, viz. *Arthrinium* sp. (anamorph of *Apiospora montagnei* Saccardo), *Cladosporium cladosporioides* (Fresenius) de Vries, *Fusarium solani* (Martius) Appel et Wollenweber, *Gliocladium roseum* (Link) Thom, *Penicillium glabrum* (Wehmer) Westling, *P. citrinum* Thom, *Pestalotiopsis* spp., *Phoma* spp., *Trichoderma hamatum* (Bonorden) Bainier aggr., *T. harzianum* Rifai aggr., *T. koningii* Oudemans aggr., *T. viride* Persoon ex S.F. Gray aggr., *Trichoderma* sp.1, *Verticillium psalliotae* Treschow (the other Ascomycota), *Absidia glauca* Hagem, *Mortierella globurifera* Rostrup, *M. isabellina* Oudemans et Koning, *M. ramanniana* (Möller) Linneman (var. *ramanniana*), *M. verticillata* Linneman, *Mucor hiemalis* Wehmer, and *M. racemosus* Fresenius (the Zygomycota).

A successional trend was observed in species composition of 21 species in the other Ascomycota and the Zygomycota during decomposition (Fig. 4.11), and the similarities of their occurrence patterns were examined by cluster analysis (Fig. 4.12). Three large groups are identified and were termed Group I, II, and III. The decomposition stage was more important for this grouping than the site. Group I included 6 species: *Pestalotiopsis* spp., *C.*

Table 4.2 Frequency (%) of fungi isolated by surface sterilization method from beech leaf litter in litter bags collected at November 1997 (11th month).

Fungus	Upper	Lower
Geniculosporium sp.1	26.7	43.3
Xylaria sp.	16.7	46.7
White sterile mycelia	30.0	10.0
Arthrinium phaeospermum	6.7	20.0
Eupenicillium sp.	3.3	6.7
Ascochyta sp.	3.3	3.3
Coelomycete S4R171	3.3	0.0
Dactylaria obtriangularia	3.3	0.0
Discula sp.	3.3	0.0
Nodulisporium sp.	3.3	0.0
Dark sterile DIM	0.0	6.7
Mortierella globurifera	0.0	6.7
Coelomycete S4B144	0.0	3.3
Penicillium sp.4	0.0	3.3

Table 4.3 Mean frequency (%) of fungi isolated by washing method from beech leaf litter during the decomposition (n=10).

Fungus	Upper	Lower
Trichoderma koningii	29.0	42.3
Mortierella ramanniana var. *ramanniana*	22.3	35.7
Trichoderma hamatum	28.3	20.0
Gliocladium roseum	24.0	22.7
Pestalotiopsis spp.	31.7	14.0
Mucor hiemalis	12.3	23.7
Penicillium citrinum	14.7	18.3
Trichoderma sp.1	13.0	15.7
Trichoderma viride	14.0	13.7
Cladosporium cladosporioides	12.3	7.7
Mortierella globurifera	6.0	10.7
Mortierella isabellina	6.7	9.0
Mucor racemosus	6.0	5.7
Verticillium psalliotae	5.7	4.3
Phoma spp.	4.7	7.3
Penicillium glabrum	4.0	5.7

Table 4.3 Continued.

Arthrinium state of *Apiospora montagnei*	5.3	4.0
Mortierella verticillata	2.7	6.0
Absidia glauca	4.7	4.0
Fusarium solani	2.0	6.3
Trichoderma harzianum	2.3	6.0
Penicillium thomii	3.3	10.7
Trichoderma pseudokoningii	5.7	6.0
Gliocladium virens	5.0	2.7
Trichoderma polysporum	4.3	2.3
Rhizopus cf. *rhizopodiformis*	3.0	0.3
Acremonium sp.2	2.7	2.3
Chaetomium globosum	2.7	0.3
Aspergillus sp.	2.7	0.3
Penicillium velutinum	2.3	5.0
Aspergillus japonicus	2.0	2.7
Epicoccum nigrum	2.0	1.0
Cladosporium herbarum	2.0	0.3
Mortierella ramanniana var. *angulispora*	1.7	3.0
Paecilomyces carneus	1.7	1.3
Verticillium cf. *suchlasporium*	1.3	4.0
Mucoraceae 1	1.3	0.7
Penicillium melinii	1.3	0.7
Coniothyrium sp.	1.0	2.0
Gliocladium viride	1.0	1.7
Alternaria alternata	1.0	1.3
Verticillium chlamydosporium	1.0	1.0
Penicillium chrysogenum	1.0	0.3
Scedosporium sp.	1.0	0.0
Mucor sp.	0.7	1.3
Acremonium sp.3	0.7	0.7
Cladosporium sp.	0.7	0.7
Colletotrichum sp.	0.7	0.3
Acremonium sp.1	0.7	0.0
Trichoderma sp.2	0.3	1.7
Volutella ciliata	0.3	1.7
Penicillium montanense	0.3	1.3

Table 4.3 Continued.

Mortierella sp.1	0.3	1.0
Cladosporium oxysporum	0.3	0.7
Coelomycete AKA	0.3	0.7
Fusarium graminearum	0.3	0.3
Phialophora sp.	0.3	0.3
Absidia spinosa	0.3	0.0
Acremonium strictum	0.3	0.0
Arthrinium phaeospermum	0.3	0.0
Cladosporium erratum	0.3	0.0
Coelomycete 10R372	0.3	0.0
Coelomycete 5R112	0.3	0.0
Discula sp.	0.3	0.0
Fusarium oxysporum	0.3	0.0
Fusarium sp.	0.3	0.0
Gliomastix felina	0.3	0.0
Hypomycete 3R362	0.3	0.0
Mucoraceae 2	0.3	0.0
Penicillium citreonigrum	0.3	0.0
Penicillium sclerotiorum	0.3	0.0
Penicillium sp.1	0.3	0.0
Phialophora verrucosa	0.3	0.0
Phomopsis sp.	0.3	0.0
Penicillium janthinellum	0.0	2.0
Aspergillus kanagawensis	0.0	1.3
Mortierella sp.2	0.0	1.0
Fusarium cf. *redolens*	0.0	0.7
Mortierella minutissima	0.0	0.7
Mortierella wuyshanensis	0.0	0.7
Penicillium lividum	0.0	0.7
Absidia cylindrospora	0.0	0.3
Acremonium sp.4	0.0	0.3
Ascochyta sp.	0.0	0.3
Ascomycete 3B375	0.0	0.3
Calcarisporium arbuscula	0.0	0.3
Chaetomium sp.	0.0	0.3
Chrysosporium sp.	0.0	0.3

Table 4.3 Continued.

Coelomycete 1B1101	0.0	0.3
Geniculosporium sp.2	0.0	0.3
Hyphomycete 2B153	0.0	0.3
Mortierella autotrophica	0.0	0.3
Mucor plumbens	0.0	0.3
Paecilomyces farinosus	0.0	0.3
Paecilomyces sp.2	0.0	0.3
Penicillium corylophilum	0.0	0.3
Penicillium miczynskii	0.0	0.3
Penicillium sp.2	0.0	0.3
Penicillium sp.3	0.0	0.3
Rhizopus sp.	0.0	0.3
Trichocladium asperm	0.0	0.3
Trichoderma piluliferum	0.0	0.3
Dark sterile mycelia	0.7	0.0
White sterile mycelia	2.3	1.0

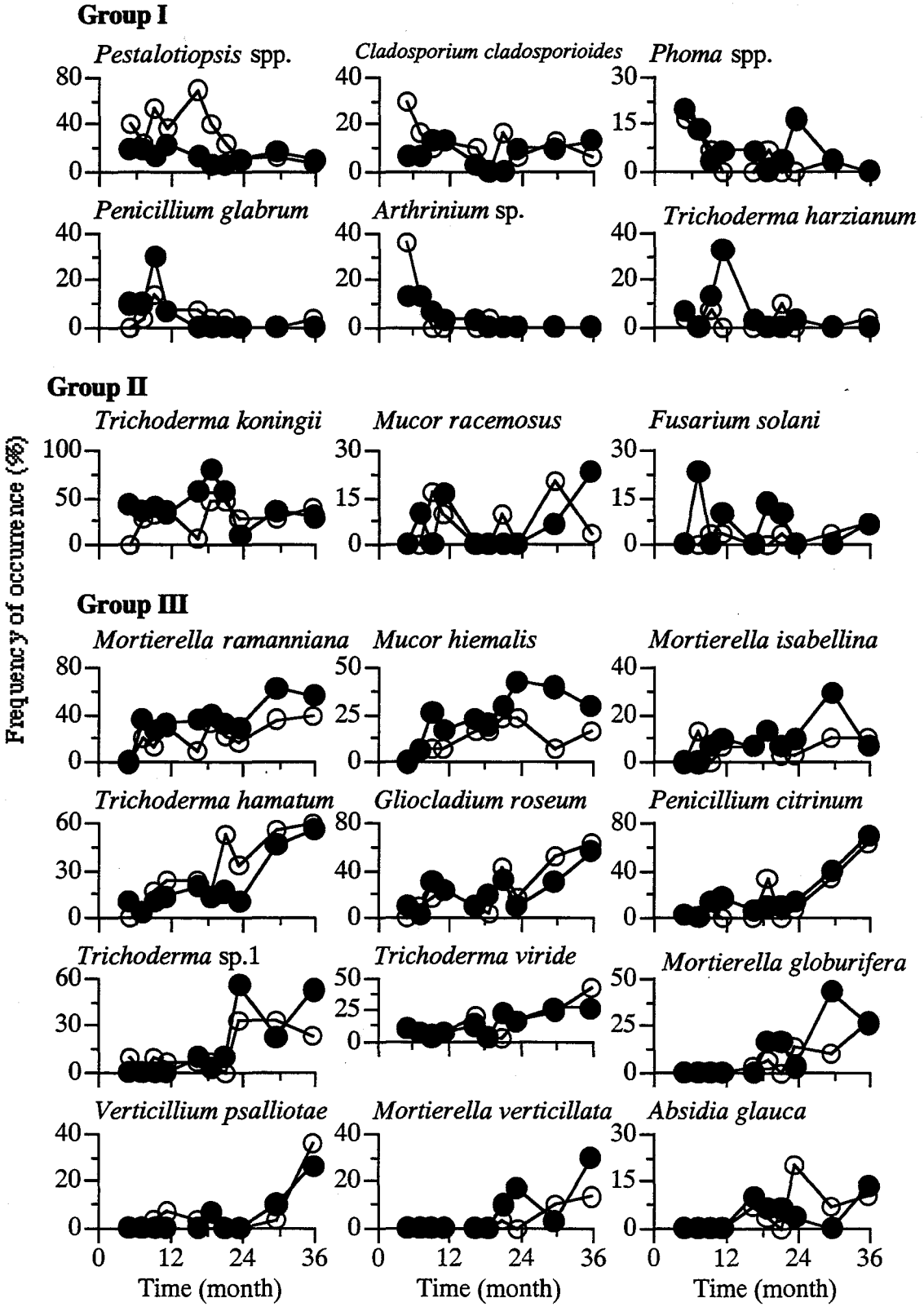

Fig. 4.11 Changes in frequency of 21 species in the other Ascomycota than the Xylariaceae and in the Zygomycota isolated by the washing method from beech leaf litter during decomposition. Groups are shown in the cluster analysis. ○ = upper, ● = lower.

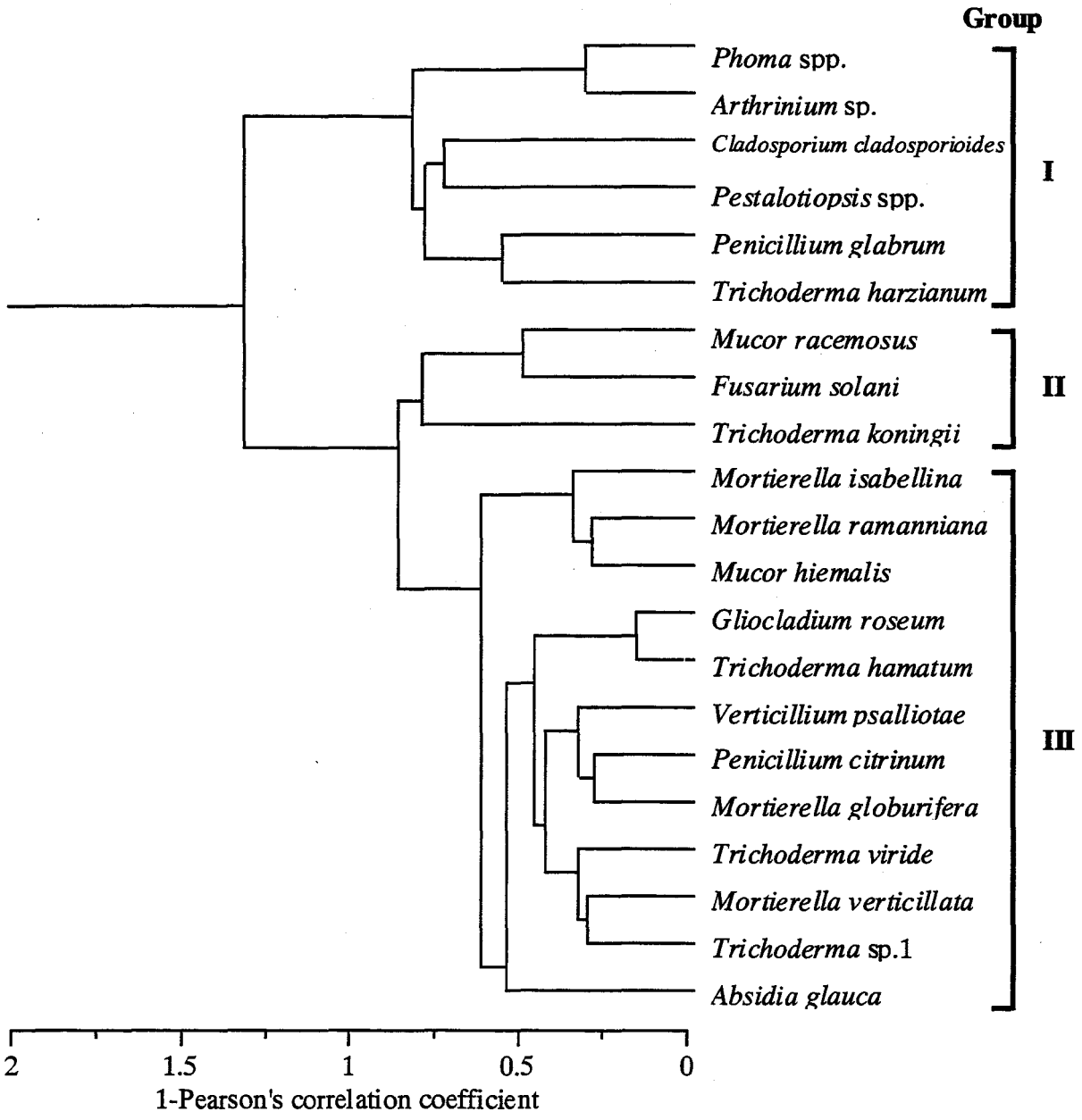

Fig. 4.12 Dendrogram of 21 species in the other Ascomycota than the Xylariaceae and in the Zygomycota by the group-average method with Pearson's correlation coefficient based on occurrence time and site. Groups identified are depicted by parentheses.

Table 4.4 Correlation coefficients for linear relations between LCI and soluble carbohydrate concentration of beech leaf litter and frequency 21 frequent fungi during decomposition (n=11).

Fungus	LCI		Soluble carbohydrate	
	Upper	Lower	Upper	Lower
Group I				
Pestalotiopsis spp.	0.454	0.572	0.736**	0.489
Cladosporium cladosporioides	0.500	0.036	0.415	-0.036
Phoma spp.	0.735**	0.609*	0.554	0.501
Penicillium glabrum	0.191	0.735*	0.317	0.792**
Arthrinium sp.	0.708*	0.852***	0.571	0.893***
Trichoderma harzianum	0.116	0.505	0.083	0.461
Group II				
Trichoderma koningii	-0.447	0.131	-0.457	0.240
Mucor racemosus	-0.112	-0.334	-0.172	-0.337
Fusarium solani	-0.289	-0.161	-0.427	0.061
Group III				
Mortierella ramanniana	-0.626*	-0.794**	-0.677*	-0.687*
Mucor hiemalis	-0.806**	-0.784**	-0.604*	-0.741**
Mortierella isabellina	-0.477	-0.616*	-0.464	-0.671*
Trichoderma hamatum	-0.870***	-0.621*	-0.837**	-0.810**
Gliocladium roseum	-0.674*	-0.473	-0.768**	-0.617*
Penicillium citrinum	-0.496	-0.675*	-0.726*	-0.815**
Trichoderma sp.1	-0.447	-0.818**	-0.616*	-0.896***
Trichoderma viride	-0.544	-0.489	-0.715*	-0.791**
Mortierella globurifera	-0.792**	-0.724*	-0.908***	-0.839**
Verticillium psalliotae	-0.402	-0.728*	-0.551	-0.731*
Mortierella verticillata	-0.621*	-0.701*	-0.780**	-0.831**
Absidia glauca	-0.781**	-0.620*	-0.786**	-0.560

Groups are shown in the cluster analysis. Frequency was arcsin-transformed and used for calculation.

* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$. LCI = lignocellulose index.

cladosporioides, *Phoma* spp., *P. glabrum*, *Arthrinium* sp., and *T. harzianum*. They were the early occurring species whose frequency was high during the immobilization phase and then decreased as the decomposition progressed. Group II included 3 species: *T. koningii*, *M. racemosus*, and *F. solani*. They occurred constantly throughout the decomposition. Group III included 12 species: *M. ramanniana*, *M. hiemalis*, *M. isabellina*, *T. hamatum*, *G. roseum*, *P. citrinum*, *Trichoderma* sp.1, *T. viride*, *M. globurifera*, *V. psalliotae*, *M. verticillata*, and *A. glauca*. They were the late occurring species whose frequency increased during the mobilization phase.

Table 4.4 shows correlation coefficients for linear relations between LCI and soluble carbohydrate concentration of beech leaf litter and frequency of 21 species. They showed different responses to LCI and soluble carbohydrate concentration of the litter between the groups. Frequencies of *Pestalotiopsis* spp., *Phoma* spp., *P. glabrum*, and *Arthrinium* sp. in Group I were significantly ($p<0.05$) and positively related to LCI and/or soluble carbohydrate concentration in the upper or the lower site, or in both sites. Frequencies of *C. cladosporioides* and *T. harzianum* in Group I and frequencies of three species in Group II were not significantly related to the resource quality in both sites. Frequencies of 12 fungi in Group III were significantly ($p<0.05$) and negatively related to LCI and/or soluble carbohydrate concentration in the upper or the lower site, or in both sites.

Discussion

In cool temperate deciduous forests in Japan, moder and mull humus develops on the upper and lower part of forest slopes, respectively (Takeda and Kaneko 1988). Takeda et al. (1987) reported that litter decomposition rate is higher in mull humus than in moder humus, as in the central Europe deciduous forests where mull humus often develops in calcareous soils and mor or moder humus develops in acidic sandy soils (Swift et al. 1979). In this study, however, decomposition rates of beech leaf litter were similar between the sites. This result is consistent with Kaneko (1995) who reported the decomposition processes of beech leaf litter were similar between the sites. As is pointed out by Kaneko (1995), this may be because of the use of the litter bags of fine mesh which inhibited a colonization of macrofaunal decomposers into the litter bags. The litter bags used in this study thus provided a similar habitat for decomposer fungal populations live in different soil types.

Decomposition processes of beech leaf litter showed two phases: (i) nitrogen and phosphorus immobilization (0-21 months); and (ii) mobilization (21-35 months). Net increase in nitrogen and phosphorus weights during the immobilization phase can be ascribed to high initial lignin content and low initial nitrogen and phosphorus content of beech leaf litter according to Aber and Melillo (1982). Net weight loss of nitrogen and phosphorus occurred when C/N and C/P approached to asymptotes during the mobilization phase. Takeda et al. (unpublished) have already found that, irrespective of the initial values, C/N of leaf litter of 18 tree species converged at 20-30 (25 on the mean) during a 20 month decomposition period and that net nitrogen mobilization started at the critical C/N values. The result that immobilization of phosphorus was higher in the lower site than in the upper site may be due to high exogenous phosphorus supply to the decomposing beech leaf litter in the lower site compared to the upper site (Melillo and Aber 1984). Weight changes in potassium, calcium, and magnesium showed only mobilization phase. This is consistent with previous studies on needle litters (Staaf and Berg 1982; Hasegawa and Takeda 1996) and broad-leaved litters (Gosz et al. 1973; Blair 1988).

The order of loss of organic chemical constituents was lignin < holocellulose < soluble carbohydrate < polyphenol in order. Berg et al. (1982) also reported that water soluble components showed faster weight losses than structural components such as holocellulose and lignin. The result that the changes in LCI during decomposition were characterized by two phases is consistent with Berg et al. (1984) and Melillo et al. (1989). The changes in LCI were significantly correlated to the changes in nitrogen and phosphorus concentrations during decomposition, indicating that nitrogen and phosphorus dynamics were related to the changes in relative availability of holocellulose in lignocellulose matrix. During the immobilization phase, high availability of holocellulose leads to the rapid fungal ingrowth during the first year that contributed to nitrogen and phosphorus immobilization. There have been two explanations for the mechanism of fungal immobilization of nitrogen and phosphorus. The first is that a major part of the total nitrogen and total phosphorus in the litter should be retained by fungal biomass. This does not, however, appear to apply here, because even when the fungal biomass in the litter was highest, the amount of nitrogen bound in the fungal biomass only made up 1.5% (upper site, 11th month) and 1.8% (lower site, 7th month) of the total litter nitrogen and that of phosphorus made up 7.5% (upper) and 7.8% (lower) of the total litter phosphorus, assuming nitrogen content of 3.7% and phosphorus content of 0.7% of fungal biomass (Bååth and Söderström 1979). Berg and Söderström (1979) also reported that the increase of nitrogen bound in fungal biomass is not enough to explain the increase in total nitrogen amount in decomposing pine needle litter. The second is that fungal ingrowth may cause nitrogen to be retained in the litter as recalcitrant compounds such as protein-lignin complexes (Berg 1986, 1988). This hypothesis is supported by our unpublished finding (Osono et al. unpublished) that content and absolute amount of nitrogen in lignin fraction of beech litter increased during the immobilization phase. In the mobilization phase, on the other hand, LCI reached asymptotes and the decomposition processes may be controlled by the availability of carbon sources derived from the decomposition of refractory compounds such as lignin, lignin-like nitrogenous humic substances, and lignified holocellulose. Concurrently, fungal ingrowth decreased. With the low availability of carbon energy sources to fungal

populations, thus, the mobilization of nitrogen and phosphorus may exceed the immobilization and these elements were released from the litter during the mobilization phase.

Lignin to nutrient ratios followed a similar pattern to carbon to nutrient ratios. L/N decreased as the immobilization of nitrogen into litter during the first 21 months and then nitrogen started to be released from the litter when L/N reached at about 26-27. This indicates that nitrogen can be incorporated into lignin until the lignin is saturated with nitrogen at the ratio of about 25. Aber and Melillo (1982) also reported total nitrogen immobilization into litter was expressed as a function of its lignin content. Osono and Takeda (unpublished) found the critical values of L/N of 14 tree species converged at 20 on mean irrespective of the initial values.

The proportion of clamp-bearing fungal biomass to total fungal biomass increased as the decomposition proceeded. Some authors also reported that the density of basidiomycetous mycelia was higher in the F layer than in the upper L layer (Saito 1956; Bååth and Söderström 1977; Frankland 1982). The negative relationship between LCI and the percentage of clamp-bearing fungal biomass indicate that basidiomycetous fungi preferentially colonized on the litter with the low availability of carbon energy sources. The ability of the Basidiomycota to decompose lignin is the highest among litter decomposing fungi (Lindeberg 1944, 1946; Saito 1960; Hering 1967, 1982; Miyamoto et al. 2000; Osono and Takeda 2002a). They decomposed lignin and lignin-like humic substances vigorously in forest soils to produce "white-rot humus" (Harris 1945; Saito 1957; Hintikka 1970, 1982). Furthermore, concentration of exchangeable NH_4^+ and nitrogen mineralization rate during the laboratory incubation were higher in the white-rot humus than in the surrounding humus (Hintikka 1970). These suggested that litter inhabiting basidiomycetous fungi played functional roles in the simultaneous decomposition of lignin and related humic substances and lignified holocellulose and the release of nitrogen and phosphorus in the beech litter during the mobilization phase.

During the immobilization phase in the first 21 months, on the other hand, growth of

basidiomycetous hyphae was relatively low, while *Geniculosporium* sp.1 and *Xylaria* sp. (anamorph) were frequent in the 11th month litter. The predominance of the Ascomycota in the interior of litter in earlier stages of decomposition has been reported on several tree species (Hudson 1968; Tokumasu 1996). Osono and Takeda (1999b, 2001a) have already found that these xylariaceous species took part in lignocellulose decomposition in beech leaf litter. We thus suggested that these xylariaceous species may be major functional decomposers during the immobilization phase, decomposing holocellulose in preference to lignin and growing rapidly. This is supported by Osono and Takeda (2002a) that reported these xylariaceous species attack holocellulose in preference to lignin in beech leaf litter *in vitro*.

Whether *Geniculosporium* sp.1 and *Xylaria* sp. (anamorph) persisted until the mobilization phase and took part in litter decomposition is unclear. However, our data indicate that *Geniculosporium* sp.1 and *Xylaria* sp. (anamorph) caused low and negligible weight loss, respectively, when inoculated under laboratory condition to 'mobilization phase' beech litter (LCI=0.34) compared to freshly fallen beech litter (LCI=0.45) (see Chapter 5). We thus considered that the litter decomposing activity of *Geniculosporium* sp.1 and *Xylaria* sp. (anamorph) would be lower and/or they would be less frequent during the mobilization phase than the immobilization phase. It is thus speculated that selective holocellulose decomposition by these xylariaceous species changed the resource quality that was unsuitable for themselves but suitable for the Basidiomycota, leading to the successive replacement of the functional group from the Xylariaceae to the Basidiomycota.

A successional trend was observed in the composition of 21 frequent species in the other Ascomycota and the Zygomycota during decomposition. The successional pattern is similar to that reviewed in Hudson (1968) on decomposing litter of several tree species. These species were classified into 3 groups based on their occurrence patterns. As these species had a limited ability to attack lignin and depended on non-lignified holocellulose or soluble carbohydrate for their growth (Osono and Takeda 2002a), the changes in their frequencies were related to the changes in LCI and soluble carbohydrate concentration during

decomposition.

The decrease of frequency of 4 species in Group I was significantly correlated to the decrease in LCI and soluble carbohydrate concentration. This suggested that they depended on non-lignified holocellulose or soluble carbohydrate of plant origin for their growth. They are regarded as 'primary saprophytes' sensu Hudson (1968) persisting from the phyllosphere of beech leaves (Osono 2002) and gaining first access to these resources.

Frequencies of 3 species in Group II were not significantly related to the resource quality in both sites. They were considered as 'litter inhabitants' colonizing the litter of various resource availability. Factors other than the resource availability such as temperature or moisture may affect the occurrence of these species on the decomposing litter.

Twelve fungi in Group III were the late occurring species whose frequency increased as the decrease in LCI and soluble carbohydrate concentration, suggesting that they depend for their growth on sugars released from the holocellulose fraction by ligninolytic activity of functional species in the Basidiomycota and the Xylariaceae. Saito (1965) and Hudson (1968) called these fungi 'secondary sugar fungi' that occurred in association with lignocellulose decomposers.

In conclusion, the organic chemical, nitrogen, and phosphorus dynamics during decomposition of beech leaf litter were related to the ingrowth, substrate utilization, and succession of lignocellulose decomposers in the Xylariaceae and the Basidiomycota (Table 4.5). These species are thus regarded as functional species that decompose holocellulose and lignin in the litter structure and play a major role in beech litter decomposition. Twenty-one species in the other Ascomycota and the Zygomycota, on the other hand, are regarded as associated species that depend for their growth on non-lignified holocellulose or soluble carbohydrate that constitute only a small portion of beech litter and play only a minor role in litter weight loss. Mycelial abundance of these species may be low regardless of their high frequencies. They were classified into three groups based on their occurrence patterns: primary saprophytes, litter inhabitants, and secondary sugar fungi. They showed different responses to LCI and soluble carbohydrate concentration of the litter between the groups.

The present study illustrates the importance of understanding the effects and interactions of specific functional groups, rather than assumptions about the functional competence of diverse communities, on the process of litter decomposition. Cox et al. (2001) reached at the similar conclusion who studied the effects of fungal inoculation on the decomposition of lignin and polysaccharides in *Pinus* litter.

Table 4.5 Relation of fungal ingrowth and succession with characters of two phases.

Decomposition phase	Immobilization phase	Mobilization phase
Time (months)	0-21	21-35
Nitrogen and phosphorus	immobilization	mobilization
C/N	55 –> 26	26 –> 24
C/P	1340 –> 760 (ridge) 610 (bottom)	760 –> 680 (ridge) 610 –> 540 (bottom)
LCI	0.45 –> 0.34	0.34 –> 0.33
Fungal ingrowth	high	low
Fungal succession		
i) functional species: lignocelulose decomposers	Xylariaceous Ascomycota *Geniculosporium* sp.1 *Xylaria* sp.	Basidiomycota mainly *Mycena* spp.
- Function	- Selective holocellulose decomposition	- Simultaneous decomposition of holocellulose and lignin
ii) associated species:	Other Ascomycota & Zygomycota	
cellulose decomposers and sugar fungi	'Primary saprophytes' (Group I) *Pestalotiopsis* spp. *Phoma* spp. *Penicillium glabrum* *Arthrinium* sp. *Cladosporium cladosporioides* *Trichoderma harzianum*	'Secondary sugar fungi' (Group III) *Mortierella ramanniana* *Mucor hiemalis* *Mortierella isabellina* *Trichoderma hamatum* *Gliocladium roseum* *Penicillium citrinum* *Trichoderma* sp.1 *Trichoderma viride* *Mortierella globurifera* *Verticillium psalliotae*
	'Litter inhabitants' (Group II) *Trichoderma koningii* *Mucor racemosus* *Fusarium solani*	
- Function	- Decomposition of non-lignified holocellulose and soluble carbohydrate	

The immobilization phase from 0 to 21 month and the mobilization phase from 21 to 35 month, in terms of nitrogen and phosphorus state, C/N, C/P, and lignocellulose index (LCI).

Chapter 5

Comparison of litter decomposing ability among diverse fungi

Introduction

Fungi play fundamental roles in decomposition processes of leaf litter within forest ecosystems (Swift et al. 1979; Cooke and Rayner 1984). Fungal species composition and successional changes during the decomposition process have been qualitatively investigated on several litter types (Hudson 1968), but the frequency of occurrence of individual species is a poor guide to their importance in the decomposition processes. Pure culture decomposition tests have been therefore carried out to assess the decomposing abilities and the substrate utilization patterns of fungi (Lindeberg 1944, 1946; Mikola 1956; Saito 1960; Hering 1967; 1972; De-Boois 1976; Dix and Simpson 1984; Kuyper and Bokeloh 1994; Osono and Takeda 1999b).

Lignin and holocellulose are structural components that constitute 70-80% of fresh organic material and major energy sources in plant tissues that are available to fungi (Swift et al. 1979). Many studies have reported that the Basidiomycota account for most of the lignocellulose decomposition in leaf litter (Lindeberg 1944, 1947; Hering 1967; Dix and Simpson 1984; Miyamoto et al. 2000; Steffen et al. 2000). In addition, Osono and Takeda (1999b) reported that fungi in the Xylariaceae (Ascomycota) that colonize the interior of leaf tissues have the ability to decompose lignin and carbohydrate in beech litter. Because decomposition of lignin and holocellulose are key factors controlling litter decomposition rates (Aber et al. 1990), it is important to evaluate the lignin and cellulose decomposing ability of fungi occurring on litter in order to understand their roles in decomposition processes (Lindeberg 1946; Saito 1960; Hering 1967; Kuyper and Bokeloh 1994; Osono and Takeda 1999b; Miyamoto et al. 2000). However, in contrast to wood decomposition (e.g. Otjen et al. 1987; Rayner and Boddy 1988; Nilsson et al. 1989; Tanesaka et al. 1993; Worrall

et al. 1997), few surveys have been carried out comparing the leaf litter decomposing ability of diverse fungi.

Nitrogen and phosphorus dynamics in decomposing litter show leaching, immobilization and mobilization phases, while potassium, calcium and magnesium released from litter, showing no net immobilization phase (Berg and Staaf 1981; Staaf and Berg 1982; Osono and Takeda 2001b). Immobilization indicates the retention, or net increase, of nutrients in the litter and mobilization indicates the net release of nutrients. Meanwhile, studies on fungal succession during the litter decomposition have shown that colonization of fungal populations is related to these phases (Deka and Mishra 1982; Slapokas and Granhall 1991; Robinson et al. 1994; Osono and Takeda 2001b). Nutrient analyses in litter decomposed by each fungal species under a pure culture condition will provide useful information for the understanding of the role of each fungal population in nutrient dynamics during litter decomposition. However, few studies have been carried out to assess nutrient contents in litter decomposed *in vitro* by diverse fungal species.

I investigated and compared the ability of 79 fungal isolates (41 genera, 60 species) in the Basidiomycota, the Ascomycota (Xylariaceae and others), and the Zygomycota to decompose beech leaf litter under laboratory conditions. Fungi were isolated either from fruit bodies on leaf litter, twigs, cupules, or wood; from green leaves, leaf litter, or twigs of beech; or from mineral soils. Isolates from green leaves were used to examine litter decomposing ability because some of these fungi persisted after litter fall and occurred as litter decomposers (Osono 2002). In addition, nutrient contents (N, P, K, Ca, Mg) were measured for the litter decomposed by 19 selected isolates in 15 species in the Basidiomycota and the Ascomycota (Xylariaceae and others).

Materials and Methods

Fungi

The 79 fungal isolates used in the decomposition test, date, source, and method of isolation are listed in Table 5.1. The isolates were qualitatively collected on several occasions during a 21-month period from October 1996 to June 1998 from the Ashiu Experimental Forest of Kyoto University (see Material and Study Site). Isolation were made either from mass basidiospores, a single ascospore, or mass ascospores discharged from fruit bodies on leaf litter, twigs, cupules, or wood; from green leaves, leaf litter, or twigs of beech, with the surface sterilization or the washing method (Osono and Takeda 1999b); or from mineral soils with dilution plating method (Osono and Takeda 2000). All fungal isolates were maintained on slants of a modified malt-yeast-soytone agar (Kinugawa 1988) at a room temperature (ca. 15-20°C) in darkness. Malt-yeast-soytone agar contains malt extract 0.35%, soytone 0.05%, yeast extract 0.025%, and agar 1.5% (w/v).

Decomposition test

The ability of isolates to decompose beech leaf litter was assessed by the pure culture decomposition test (Osono and Takeda 1999b). Leaf litter used in the test was collected from the study area by litter-traps in autumn 1997. Leaf disks, including the primary vein, were punched out with a cork borer (20 mm in diameter). A subsample of seven disks were air-dried at 40°C for 4 days and weighed to obtain the original weight of the leaf disks.

Leaf disks were pressed in moistened paper towels between the base and lid of a Petri dish, then autoclaved at 120°C for 20 min. The sterilized disks were placed on surfaces of Petri dishes containing 20 mL 2% plain agar. Inocula for each assessment were cut out of the margin of the growing colonies on 2% malt extract agar (Hawksworth et al. 1995) with a sterile cork borer (5.5 mm in diameter) and placed on the center of the plates including a subsample of seven disks around the inoculum. Plates were incubated for 8 weeks at 20°C in darkness.

After 8 weeks leaf disks were collected, oven dried for 4 days at 40°C, and weighed. Observations were made of the external appearance of the decomposed litter, and bleaching (i.e. change in color from brown to white) was observed under a binocular microscope with a magnification of 20x. Weight loss of the leaf disks was determined as a percentage of the original weight. Ten plates were prepared for each strain. A portion of the sample leaves were used for chemical analyses as described below.

Chemical analyses

The amount of lignin in samples was estimated by gravimetry using hot sulfuric acid digestion (King and Heath 1967). Total carbohydrate was estimated by the phenol-sulfuric acid method (Dubois et al. 1956) according to the method described in Fukui (1969). The methods are described in Chapter 2.

Mean concentrations of lignin and carbohydrate in the initial litter were 39.6% and 34.2%, respectively. Weight losses of lignin and carbohydrate were expressed as percentage of the original weights.

Lignin/weight loss ratio (L/W) and lignin/carbohydrate loss ratio (L/C) are useful indices of the substrate utilization pattern of each fungal isolate (Worrall et al. 1997). L/W and L/C of an isolate are calculated according to the following equations:

L/W = weight loss of lignin (% original weight of lignin) / weight loss of litter (% original weight of litter)

L/C = weight loss of lignin (% original weight of lignin) / weight loss of carbohydrate (% original weight of carbohydrate)

Total nitrogen content was measured by automatic gas chromatography (NC analyzer SUMIGRAPH NC-900, Sumitomo Chemical Co., Osaka, Japan). After an acid wet oxidation in HNO_3 + $HClO_4$, the following analyses were performed; molybdate-ascorbic acid method for phosphorus (Olsen and Sommers 1982), flame photometry for potassium and atomic

absorption for calcium and magnesium (atomic absorption spectrophotometer 170-30S, Hitachi Ltd., Tokyo, Japan).

Analysis of variance (Systat 1992) was used to determine the differences between mean values of nutrient contents among the taxa. The least significant difference test was used for multiple comparisons.

Results

Weight loss

Weight loss of beech leaf litter ranged from 0.1% to 57.6% (Table 5.1, Figs. 5.1 and 5.2). High weight losses were caused by six isolates in the Basidiomycota (*Lentinula edodes*, *Microporus vernicipes*, *Mycena polygramma*, *Mycena* sp., *Naematoloma sublateritium*, and *Panellus serotinus*) and ranged from 15.1% to 57.6%, whereas *Guepiniopsis* sp. and *Xylobolus frustulatus* caused weight losses of 4.2% and 4.3%, respectively. The mean weight loss of the Basidiomycota was 25.5%. *Xylaria* spp. and *Geniculosporium* spp. also caused high weight losses ranging from 4.8% to 14.4% and a mean weight loss for this group was 7.6%. Other Ascomycota (including anamorphs) caused weight losses ranging from 0.1% to 6.5% and a mean weight loss of 2.6%. Fungi in the Zygomycota caused weight losses ranging from 1.1% to 4.9% and a mean weight loss of 2.7%. Sterile mycelia caused weight losses ranging from 1.4% to 8.8% and a mean weight loss of 5.0%.

Bleaching was noticeable in leaf litter decomposed by six isolates in the Basidiomycota, all 14 isolates in the Xylariaceae, and two isolates of white sterile 5LS12 (Table 5.1). Mean weight loss of only bleached litter was 14.4% $\pm$ 2.9% (mean $\pm$ s.e.) and was significantly ($p<0.01$, T-test) higher than that of the non-bleached litter (2.6% $\pm$ 0.2%).

Chemical changes and substrate utilization

Weight losses of lignin and carbohydrate were measured for the litters decomposed by 13 isolates (six in the Basidiomycota, five in the Xylariaceae, and two white sterile 5LS12) that were representative of 22 isolates with the bleaching activity. Preliminary DNA analysis indicated the white sterile 5LS12 isolate belonged to the Xylariaceae, therefore decomposition results are subsumed under the Xylariaceae in the analysis.

Weight loss of lignin ranged from 6.5% to 59.8% and from 2.0% to 12.2%, in the

Table 5.1 Isolates used in the litter-decomposing test, date, source, and method, bleaching activity, and weight loss after 8 weeks (mean ± SE).

Taxa[a]	Isolation			Bleaching activity[d]	Weight loss (% original weight)
	Date	Source[b]	Method[c]		
BASIDIOMYCOTA					
Guepiniopsis sp.	Nov. '97	B (W)	SD	-	4.2 ± 0.9
Lentinula edodes (Berk.) Pegler	Apr. '98	B (W)	SD	+	57.6 ± 1.6
Microporus vernicipes (Berk.) O. Kuntze	Nov. '97	B (T)	SD	+	15.1 ± 0.6
Mycena polygramma (Bull.: Fr.) S.F. Gray	Nov. '97	B (L)	SD	+	36.6 ± 1.6
Mycena sp.	Nov. '97	B (L)	SD	+	24.0 ± 1.3
Naematoloma sublateritium (Fr.) Karst.	Nov. '97	B (W)	SD	+	23.9 ± 0.9
Panellus serotinus (Pers.: Fr.) Kühn.	Nov. '97	B (W)	SD	+	38.1 ± 1.7
Xylobolus frustulatus (Pers.: Fr.) Boidin	Nov. '97	B (W)	SD	-	4.3 ± 0.3
ASCOMYCOTA (XYLARIACEAE)					
Geniculosporium sp.1	Oct. '96	L	SS	+	7.3 ± 0.3
Geniculosporium sp.1	Oct. '96	L	SS	+	11.2 ± 0.4
Geniculosporium sp.2	Oct. '96	L	SS	+	4.8 ± 0.2
Xylaria carpophila (Pers.) Fr.	Aug. '97	A (C)	SD	+	14.4 ± 0.9
Xylaria carpophila (Pers.) Fr.	Jul. '97	A (C)	SSI	+	4.0 ± 0.6
Xylaria sp.	Oct. '96	L	SS	+	9.9 ± 0.8
Xylaria sp.	Oct. '96	L	SS	+	8.2 ± 0.4
Xylaria sp.	Oct. '96	L	SS	+	7.8 ± 0.5
Xylaria sp.	Oct. '96	L	SS	+	7.5 ± 0.5
Xylaria sp.	Aug. '97	L	SS	+	7.0 ± 0.4
Xylaria sp.	Oct. '96	L	SS	+	6.5 ± 0.3

Table 5.1 Continued.

Xylaria sp.	Aug. '97	G	SS	+	6.1 ± 0.4
Xylaria sp.	Oct. '96	L	SS	+	5.6 ± 0.6
Xylaria sp.	Oct. '96	L	SS	+	5.5 ± 0.4
ASCOMYCOTA (others)					
Acremonium sp.	Sept. '97	L	W	-	1.2 ± 0.3
Alternaria alternata (Fr.) Keissler	Jul. '97	L	DP	-	1.7 ± 0.3
Arthrinium state of *Apiospora montagnei* Sacc.	Oct. '96	L	W	-	4.1 ± 0.2
Arthrinium state of *Apiospora montagnei* Sacc.	Oct. '97	L	DP	-	3.4 ± 0.2
Arthrinium phaeospermum (Corda) M.B. Ellis	Nov. '97	L	SS	-	0.1 ± 0.2
Ascochyta sp.	Oct. '96	G	SS	-	4.7 ± 0.1
Ascochyta sp.	Oct. '96	L	SS	-	2.7 ± 0.3
Ascochyta sp.	Oct. '96	L	SS	-	2.3 ± 0.3
Ascochyta sp.	Oct. '96	L	SS	-	0.6 ± 0.4
Aspergillus kanagawensis Nehira	May '98	S	DP	-	2.2 ± 0.3
Aspergillus japonicus Saito	Nov. '97	L	W	-	2.0 ± 0.3
Chaetomium globosum Kunze ex Steud.	Nov. '97	L	SS	-	2.4 ± 0.2
Chaetomium sp.	May '97	L	W	-	2.0 ± 0.2
Cladosporium cladosporioides (Fres.) de Vries	Oct. '96	L	W	-	1.2 ± 0.2
Colletotrichum sp.	Nov. '97	L	W	-	2.5 ± 0.3
Coniothyrium sp.	Sept. '97	L	W	-	0.7 ± 0.2
Discosia sp.	Oct. '96	L	W	-	6.5 ± 0.5
Discula sp.	Oct. '96	L	W	-	4.6 ± 0.2
Discula sp.	Oct. '96	L	SS	-	0.6 ± 0.4
Epicoccum nigrum Link ex Link	May '97	L	W	-	2.5 ± 0.3
Fusarium sp.1	Oct. '96	G	W	-	3.0 ± 0.3
Fusarium sp.2	Oct. '96	L	W	-	2.6 ± 0.3
Gliocladium roseum Bain.	Oct. '96	L	W	-	3.2 ± 0.3

Table 5.1 Continued.

Glicladium virens Miller, Giddens & Foster	Oct. '97	G	W	-	4.7 ± 0.2
Gliocladium virens Miller, Giddens & Foster	Oct. '96	L	W	-	3.1 ± 0.3
Lachnum virgineum (Batsch.: Fr.) Karsten.	Apr. '98	A (C)	SD	-	1.1 ± 0.3
Mammaria echinobotryoides Ces.	Jul. '97	L	DP	-	4.3 ± 0.2
Nigrospora state of *Khuskia oryzae* H. Hudson	Oct. '97	G	DP	-	5.0 ± 0.2
Penicillium thomii Maire	Sept. '97	L	W	-	1.1 ± 0.1
Penicillium citrinum Thom	Oct. '97	L	DP	-	2.4 ± 0.3
Penicillium citrinum Thom	Nov. '97	L	W	-	1.7 ± 0.3
Penicillium velutinum van Beyma	May '98	S	DP	-	1.1 ± 0.2
Pestalotiopsis sp.	Oct. '96	L	W	-	5.5 ± 0.3
Pestalotiopsis sp.	Oct. '96	G	W	-	3.8 ± 0.2
Phoma sp.	Oct. '96	L	W	-	0.3 ± 0.1
Phoma sp.	Oct. '96	G	W	-	1.8 ± 1.2
Phomopsis sp.	Aug. '97	T	SS	-	3.8 ± 0.2
Trichoderma hamatum (Bonord.) Bain.	Oct. '96	L	W	-	4.6 ± 0.1
Trichoderma harzianum Rifai	Oct. '96	G	W	-	3.3 ± 0.2
Trichoderma koningii Oudem.	Oct. '96	L	W	-	1.4 ± 0.4
Trichoderma longibrachiatum Rifai	Oct. '96	L	W	-	0.2 ± 0.3
Trichoderma polysporum (Link ex Pers.) Rifai	Jul. '97	L	W	-	4.5 ± 0.5
Trichoderma viride Pers. ex Gray	Oct. '96	L	W	-	3.9 ± 0.3
Verticillium chlamydosporium Goddard	Sept. '97	L	W	-	2.0 ± 0.4
Unknown GSD1 (Hyphomycetes)	Oct. '96	G	SS	-	1.1 ± 0.3
Unknown B1 (Discomycetes)	May '98	A (L)	SD	-	2.1 ± 0.3
Unknown DS (Pyrenomycetes)	Jun. '98	A (T)	SD	-	1.1 ± 0.5
ZYGOMYCOTA					
Absidia glauca Hagem	Jun. '98	L	W	-	1.9 ± 0.2
Mortierella isabellina Oudem.	Oct. '97	L	DP	-	2.8 ± 0.3

Table 5.1 Continued.

Mortierella ramanniana var. *angulispora* (Naumov) Linnem.	Jul. '97	L	DP	-	4.9 ± 0.2
Mortierella ramanniana (Möller) Linnem. var. *ramanniana*	Sept. '97	L	DP	-	2.3 ± 0.3
Mortierella wuyshanensis Chen	Oct. '96	L	W	-	3.1 ± 0.3
Mucor hiemalis Wehmer	Oct. '97	L	DP	-	1.1 ± 0.3
STERILE MYCELIA					
White sterile 5LS12	Oct. '96	L	SS	+	8.8 ± 0.6
White sterile 5LS12	Oct. '96	G	SS	+	6.2 ± 0.3
White sterile GSN2	Oct. '96	G	SS	-	3.6 ± 0.4
White sterile GSR1	Oct. '96	G	SS	-	1.4 ± 0.2

[a]The classification follows Hawksworth et al. (1995). In this study, the Ascomycota was divided into two groups, Xylariaceae and others. The groups include their anamorphs.

[b]Source. B, basidiospores; L, leaf litter; A, ascospore(s); G, green leaf; S, soil; T, twig. Substratum in parenthesis. W, wood; T, twig; L, leaf litter; C, cupule.

[c]Method. SD, spore discharge; SS, surface sterilization; SSI, single spore isolation; W, washing; DP, dilution plating.

[d]Bleaching activity. +, present; -, absent.

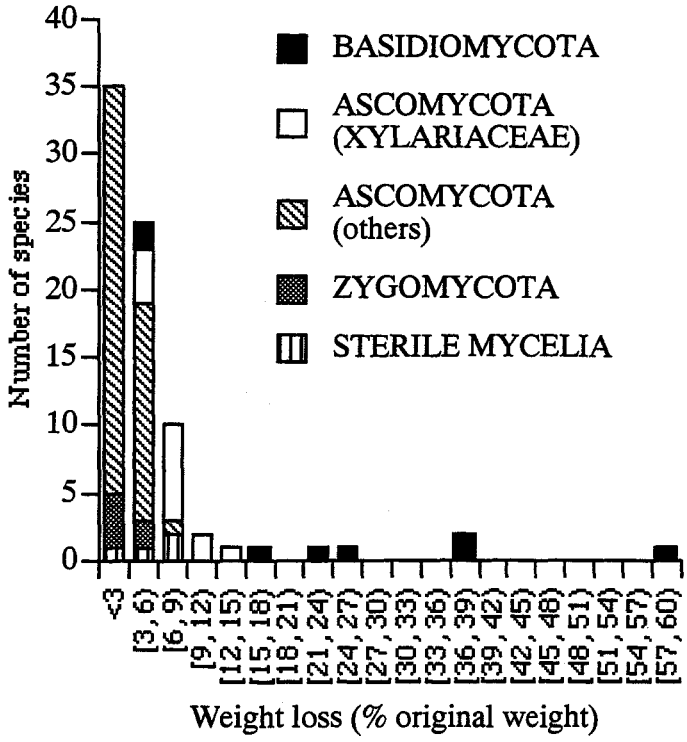

Fig. 5.1 Frequency distribution of weight loss of beech leaf litter caused by fungi.

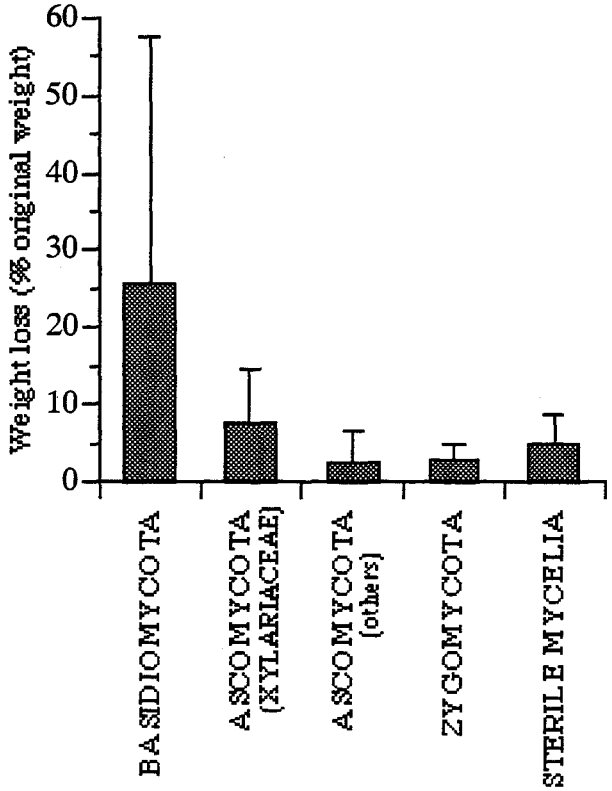

Fig. 5.2 Mean (column) and maximum (bar) weight loss of beech liter caused by fungi in each group.

Table 5.2 Weight loss of lignin and carbohydrate, lignin/litter weight loss ratio (L/W), and lignin/carbohydrate weight loss ratio (L/C) during decomposition of beech leaves after 8 weeks by fungi that exhibit bleaching activity.

Taxa	Weight loss (% original weight)		L/W	L/C
	Lignin	Carbohydrate		
BASIDIOMYCOTA				
Lentinula edodes	59.8	66.6	1.0	0.9
Microporus vernicipes	6.5	30.0	0.4	0.2
Mycena polygramma	33.2	47.8	0.9	0.7
Mycena sp.	27.0	30.2	1.1	0.9
Naematoloma sublateritium	16.3	46.0	0.7	0.4
Panellus serotinus	35.9	42.3	0.9	0.9
ASCOMYCOTA (XYLARIACEAE)				
Geniculosporium sp.1	3.6	19.9	0.5	0.2
Geniculosporium sp.1	5.0	19.9	0.8	0.3
Xylaria carpophila	3.9	29.1	0.3	0.1
Xylaria sp.	7.2	16.0	0.7	0.4
Xylaria sp.	12.2	7.6	1.5	1.6
STERILE MYCELIA				
White sterile 5LS12	2.0	23.6	0.2	0.1
White sterile 5LS12	7.6	21.5	0.7	0.4

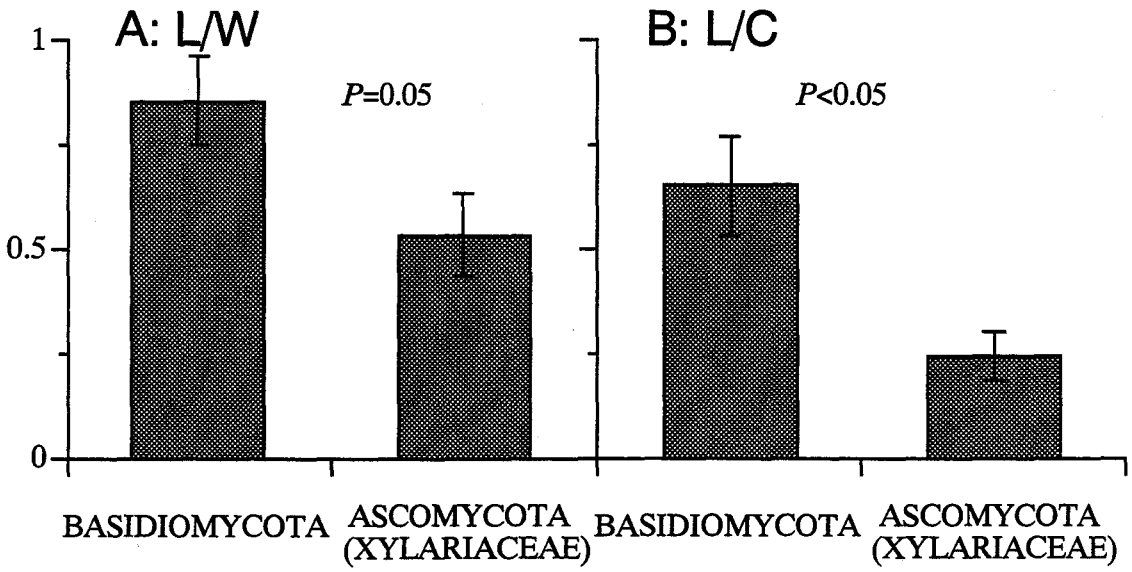

Fig. 5.3 Comparison of L/W (A) and L/C (B) between the Basidiomycota and the Xylariaceae. The result of *Xylaria* sp. which caused marked delignification was excluded in the analysis. Bars indicate standard errors.

Basidiomycota and in the Xylariaceae, respectively (Table 5.2). Weight loss of carbohydrate ranged from 30.0% to 66.6% and from 7.6% to 29.1%, respectively. L/W ranged from 0.4 to 1.1 and from 0.2 to 1.5, and L/C ranged from 0.2 to 0.9 and from 0.1 to 1.6, respectively. Variation in L/W and L/C was higher in the Xylariaceae than in the Basidiomycota, because a strain of *Xylaria* sp. showed marked delignification and high L/W and L/C.

L/W and L/C were compared between the Basidiomycota and the Xylariaceae. L/W were 0.9 $\pm$ 0.1 (mean $\pm$ s.e.) and 0.7 $\pm$ 0.2, respectively, and L/C were 0.7 $\pm$ 0.2 and 0.4 $\pm$ 0.2, respectively. No significant differences were found between two groups in L/W and L/C. However, when the result of *Xylaria* sp. that showed marked delignification was excluded in the analysis, significant differences were found in L/W (p=0.05, T-test) and L/C (p<0.05, T test) (Fig. 5.3).

Nutrient content

Table 5.3 shows weight loss and nutrient contents (N, P, K, Ca, Mg) of beech leaf litter decomposed by 19 isolates in Basidiomycota (litter B), xylariaceous Ascomycota (litter

XA) and other Ascomycota (litter OA). The nitrogen content was 1.30% in the initial litter, 1.29% in the control litter, 1.13% - 1.38% in litter B, 1.24% - 1.31% in litter XA and 1.11% - 1.25% in litter OA. The phosphorus content was 0.062% in the initial litter, 0.023% in the control litter, 0.037% - 0.053% in litter B, 0.030% - 0.075% in litter XA and 0.032% - 0.050% in litter OA. The potassium content was 0.504% in the initial litter, 0.023% in the control litter, 0.040% - 0.093% in litter B, 0.028% - 0.075% in litter XA and 0.032% - 0.071% in litter OA. The calcium content was 0.59% in the initial litter, 0.78% in the control litter, 0.65% - 0.99% in litter B, 0.67% - 0.89% in litter XA and 0.61% - 0.79% in litter OA. The magnesium content was 0.130% in the initial litter, 0.075% in the control litter, 0.028% - 0.054% in litter B, 0.073% - 0.109% in litter XA and 0.067% - 0.083% in litter OA.

Figure 5.4 shows a comparison of nutrient contents (N, P, K, Ca, Mg) in litters B, XA and OA. The mean N content in litter B was not significantly different from that in litter XA, but was significantly higher than that in litter OA. The mean P concentration in litter XA was significantly higher than that in litters B and OA. The mean Mg concentration in litter B was significantly lower than that in litters XA and OA. No significant differences were found in the mean K and Ca concentrations among the litters.

Table 5.3 Nutrient contents (N, P, K, Ca, Mg) of beech leaf litter decomposed by 19 isolates in Basidiomycota and Ascomycota (Xylariaceae and others). Weight losses represent means of ten replicates. Nutrient concentrations represent single analyses of bulked replicates.

Taxa	Concentration (%)				
	N	P	K	Ca	Mg
Initial litter	1.30	0.062	0.504	0.59	0.130
Control litter	1.29	0.023	0.023	0.78	0.075
Basidiomycota					
Panellus serotinus	1.38	0.053	0.075	0.73	0.028
Microporus vernicipes	1.27	0.037	0.040	0.75	0.054
Naematoloma sublateritium	1.36	0.053	0.087	0.67	0.029
Lentinula edodes	1.33	0.052	0.093	0.99	0.029
Mycena polygramma	1.33	0.046	0.080	0.84	0.030
Mycena sp.	1.13	0.039	0.060	0.65	0.047
Xylariaceous Ascomycota					
Xylaria carpophila	1.18	0.075	0.072	0.72	0.093
Xylaria sp.	1.26	0.056	0.058	0.76	0.088
Xylaria sp.	1.31	0.066	0.075	0.89	0.109
Geniculosporium sp.1	1.24	0.073	0.075	0.75	0.083
Geniculosporium sp.1	1.25	0.030	0.028	0.67	0.073
White sterile 5LS12	1.24	0.065	0.066	0.81	0.084
White sterile 5LS12	1.27	0.071	0.067	0.88	0.092
Other Ascomycota					
Discosia sp.	1.22	0.050	0.067	0.62	0.080
Trichoderma hamatum	1.18	0.032	0.032	0.72	0.082
Ascochyta sp.	1.25	0.046	0.071	0.76	0.076
Ascochyta sp.	1.11	0.033	0.058	0.79	0.079
Pestalotiopsis sp.	1.23	0.034	0.044	0.63	0.083
Nigrospora oryzae	1.19	0.035	0.051	0.61	0.067

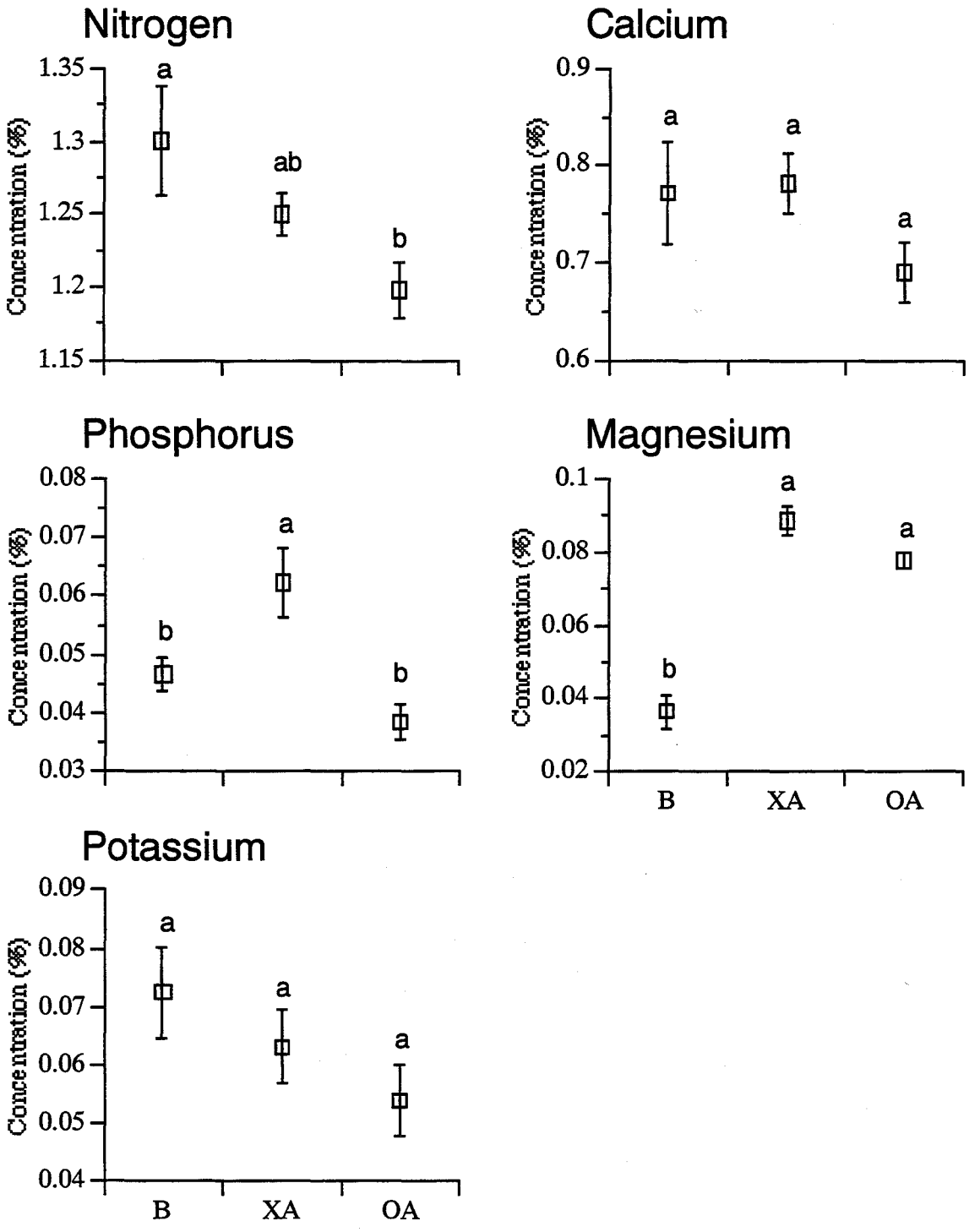

Fig. 5.4 Comparison of nutrient contents (N, P, K, Ca, Mg) in beech leaf litter decomposed by fungi in the Basidiomycota (B, n=6), Xylariaceous Ascomycota (XA, n=7) and other Ascomycota (OA, n=6). Bars indicate standard errors. The same letters indicate lack of significant difference at 5% level by least significant difference test.

Discussion

Weight loss and chemical changes

Some litter decomposing ability occurs in many groups of fungi. However, a marked decomposing ability was limited to members in the Basidiomycota and in the Xylariaceae. These fungi also had bleaching activity that was associated with lignin and carbohydrate decomposition, and were regarded as lignocellulose decomposers.

High weight losses of the litter with lignin and carbohydrate decomposition were caused by six isolates in the Basidiomycota, i.e. *Mycena polygramma*, *Mycena* sp., *Lentinula edodes*, *Microporus vernicipes*, *Naematoloma sublateritium*, and *Panellus serotinus*. *Mycena* spp. were known as vigorous decomposers of lignin and cellulose in leaf litter (Lindeberg 1946), and the others as white-rotters of wood (Imazeki and Hongo 1987, 1989; Tanesaka et al. 1993). *Mycena polygramma* and *Mycena* sp. decomposed lignin and carbohydrate in beech leaf litter as intensively as the wood fungi.

On the other hand, *Guipiniopsis* sp. and *Xylobolus frustulatus* caused lower weight losses and showed no bleaching of the litter, suggesting that some fungi in the Basidiomycota are not vigorous decomposers of beech leaf litter. Mikola (1956) also reported that decomposing abilities of basidiomycetous fungi were highly variable among species.

Fungi in the Xylariaceae caused the second highest weight loss of beech litter and lignin and carbohydrate decomposition. Xylariaceous species are known to be lignin and cellulose decomposers of wood (Merrill et al. 1964; Rogers 1979; Sutherland and Crawford 1981; Nilsson et al. 1989; Whalley 1996; Worrall et al. 1997). There have been a few studies on leaf litter decomposition by xylariaceous fungi (Osono and Takeda 1999b), but the present study showed that they caused substantial decomposition of lignin and carbohydrate. A field evidence of their bleaching activity associated with lignocellulose decomposition has already presented on the beech litter (Osono and Takeda 2001a). These thus indicate that Xylariaceous species are major decomposers of lignin and cellulose in beech leaf litter.

Other species within the Ascomycota and in the Zygomycota had low weight losses

on mean and had no bleaching activity. Among them, *Discosia* sp. and *Trichoderma hamatum* caused 22.7% and 9.3% loss of carbohydrate in the litter, respectively, and were regarded as cellulose decomposers (Osono and Takeda 1999b). Hence, some isolates that caused litter weight loss of 3.0% to 6.0%, such as *Pestalotiopsis* sp. and *Nigrospora* state of *Khuskia oryzae* may be considered as cellulose decomposing fungi. The growth of other fungi that caused weight losses below 3.0% in the test may rely mainly on readily available energy sources and be regarded as sugar fungi (Hudson 1968), as beech litter contained about 3.0% of soluble carbohydrate that fungi might consume without destroying cell wall polymers.

Substrate utilization patterns

Lignin/weight loss ratio (L/W) and lignin/carbohydrate loss ratio (L/C) are useful indices of the substrate utilization pattern of fungi. In this study, mean L/W were 0.9 and 0.7, and mean L/C were 0.7 and 0.4 for the Basidiomycota and the Xylariaceae, respectively. There has been only one comparable study of L/W and L/C of litter decomposers. In the decomposition of *Fagus sylvatica* litter by 26 basidiomycetous fungi, Lindeberg (1946) reported mean values of L/W (1.8 $\pm$ 0.5, mean $\pm$ s.d.) and L/C (1.4 $\pm$ 1.2) that were significantly ($p<0.01$, T-test) higher than those recorded in the present study. This difference is probably due to the difference in the fungal strains used and/or in the incubation method utilized.

Mean L/W and L/C of fungi in the Basidiomycota were significantly higher than in the Xylariaceae when the result of *Xylaria* sp. that caused marked delignification was excluded in the analysis. The difference in L/W and L/C between these two groups was also found in studies on decomposition of birch wood block (Otjen et al. 1987; Nilsson et al. 1989; Worrall et al. 1997). Thus, it is suggested that L/W and L/C were higher in the Basidiomycota than in the Xylariaceae in the litter decomposition and that the Xylariaceae decomposed holocellulose in preference to lignin more so than in the Basidiomycota. A strain of *Xylaria* sp. caused marked delignification. As *Xylaria* sp. occurred most frequently on bleached portions of beech litter with frequency of 60%, some strains in this species may be important in lignin

decomposition of beech litter in the study site (Osono and Takeda 2001a).

This difference may reflect the anatomical and chemical characteristics of plant cell wall decomposition by these fungi (Rayner and Boddy 1988). In the white-rot process by basidiomycetous fungi, all cell wall constituents were decomposed in the secondary wall and middle lamella (Blanchette 1995) and/or lignin was selectively removed (Otjen and Blanchette 1986). On the other hand, xylariaceous fungi caused soft-rot type decomposition by formation of cavities within the secondary wall along the microfibrillar axis or of cell wall erosion towards the middle lamella (Blanchette 1995), in which carbohydrates were preferentially attacked (Nilsson et al. 1989; Worrall et al. 1997).

Nutrient content

The nutrient dynamics of decomposing beech litter were categorized into two types (Osono and Takeda 2001b). The first type includes N and P whose weight changes were characterized by immobilization and mobilization phases. The second type includes K, Ca and Mg whose weight changes were mostly characterized by mobilization. Immobilization and mobilization dynamics of N and P were related to changes in the relative amount of lignin and holocellulose and to succession of lignocellulose decomposers (Osono and Takeda 2001b). Higher P contents in litter XA than in litter B is consistent with the trend that the xylariaceous species were dominant at the immobilization phase while basidiomycetous fungal biomass increased at the mobilization phase (Osono and Takeda 2001b). The lack of a significant difference in N contents between litters B and XA suggests that the organic chemical composition influenced more strongly N dynamics during litter decomposition than species composition of the decomposer fungi. It is interesting to note that the Mg contents differed significantly between litters B and XA in spite of the small fungal effect on Mg dynamics in decomposing litter (Osono and Takeda 2001b).

The difference in N, P and Mg contents among litters B, XA and OA is difficult to explain. These differences may be ascribed to the heterogeneous distribution of these elements within dead plant tissues and to difference in mode of tissue attack specific to each

taxon, i.e., the Basidiomycota caused the white-rot type decomposition while the Xylariaceae caused the soft-rot type decomposition (Rayner and Boddy 1988).

The decrease in P, K, and Mg contents from the initial litter to control litter is probably due to the leaching of elements during autoclaving and leaching during incubation on plain agar. Loss of K was most noticeable because of its high mobility. Other sterilization methods that impose less artificial alteration than autoclaving, such as gas sterilization or gamma irradiation, should be used for more reliable measurement of nutrient contents in litter decomposed *in vitro* by fungi.

Chapter 6

Fungal decomposition of leaf litter with different substrate quality and under different nutritional conditions

Introduction

In temperate regions, holocellulose in litter structure was preferentially utilized over lignin during the initial decomposition processes (Berg 1986; Mellilo et al. 1989; Aber et al. 1990). As a result, lignin and lignin-like recalcitrant substances remained in litter that are not readily available to most decomposer organisms. The exception is litter decomposing Basidiomycota that have an ability to lignin as well as cellulose (Lindeberg 1944, 1947; Hering 1967; Miyamoto et al. 2000) by the production of laccases and Mn-peroxidases (Steffen et al. 2000). These fungi can also attack the humus-like substances with the ligninolytic enzymes (Blondeau 1989). Experiments are needed to assess the ability of the Basidiomycota and other fungi to attack lignin and related recalcitrant substances remaining in previously partly decomposed litter

Phyllosphere fungi include those fungi that colonize the interior and surface of living leaves (Petrini 1991). Mycological studies have found that phyllosphere fungi also occur on fallen leaves of various plant species at the initial stage of decomposition (Hudson 1968; Osono 2002). Functionally some phyllosphere fungi, called 'primary saprophytes' by Hudson (1968), utilize readily available carbohydrates, and others such as xylariaceous endophytes degrade cellulose more selectively than lignin (Osono and Takeda 2002a). According to the resource utilization by phyllosphere fungi, litter decomposition progressed along with fungal succession on the litter (Hudson 1968; Osono and Takeda 2001b). The successive change from phyllosphere species to litter and soil mycobiota during decomposition suggests that the changes in litter quality due to prior colonization and consumption of available resources by phyllosphere fungi affected the growth, substrate utilization, and litter decomposition by

succeeding fungi. Especially white rot species in the Basidiomycota, vigorous decomposers of lignocellulose, may be physiologically adapted to the effective removal of lignin and related recalcitrant substances remaining in litter previously partly decomposed by phyllosphere fungi. The effects of pretreatment of wood by wood-inhabiting fungi on its subsequent decomposition by another fungus have been reported (Tanaka et al. 1988). However, such effects have not been investigated for litter-inhabiting fungi.

Prior decomposition by fungi altered organic the availability of carbon and nitrogen to succeeding fungi. Regulatory effects of carbon and nitrogen nutrition on lignin decomposition have been extensively studied in wood decomposing fungi. Most of polysaccharides in wood are protected from enzymatic attack by lignification and must be delignified for carbohydrate assimilation. This delignification depends on the availability of non-lignified carbon energy sources (Kirk et al. 1976; Reid 1991). In addition, nitrogen represses lignin decomposition, directly by interference with synthesis of ligninolytic enzyme system (Keyser et al. 1978), and indirectly by stimulating carbohydrate consumption which results in a rapid exhaustion of non-lignified carbohydrates necessary for lignin decomposition (Reid 1991). Comparative information was, however, rare on the effects of organic chemical quality and inorganic nitrogen addition on lignin decomposition by leaf litter decomposing fungi.

Three laboratory experiments were carried out to investigate the decomposition of leaf litter with different substrate quality and under different nutritional conditions. In the first experiment, the decomposition of freshly fallen litter and partly decomposed litter of beech was evaluated. In the second experiment, the effect of prior decomposition of leaf litter by phyllosphere fungi on substrate utilization by saprophytic fungi in the Basidiomycota, the Ascomycota, and the Zygomycota, was examined. In the third experiment, the effects of litter organic chemical quality and exogenous inorganic nitrogen addition on lignin decomposition were investigated.

Materials and Methods

Experiment 1: Decomposition of litter in different decay stages

Source of fungi and litters

Eight fungal species were used in this experiment: *Clitocybe* sp., *Mycena polygramma*, *Geniculosporium* sp.1, *Xylaria* sp. (anamorph), *G. serpens*, *Chaetomium globosum*, *Calcarisporium arbuscula*, and *Mortierella ramanniana* var. *ramanniana*. These fungi were collected from the Ashiu Experimental Forest of Kyoto University (see Material and Study Site). *Clitocybe* sp. and *M. polygramma* caused bleaching on litter materials on forest floor (Chapter 3). *Geniculosporium* sp.1 and *Xylaria* sp. were phyllosphere fungi that produced bleaching spots on beech leaves (Chapter 2). *G. serpens* and *C. globosum* were isolated frequently and exclusively from the bleached litter produced by *Clitocybe* sp. (Chapter 3). *C. arbuscula* and *M. ramanniana* were isolated from the bleached and non-bleached litter (Chapter 3).

Decomposition test

An *in vitro* decomposition test was carried out to assess the decomposing activity of fungi. Two litter types were used in the test: (i) freshly fallen leaves of beech collected from forest floor in autumn 1999 and (ii) partly decomposed litter exposed to natural microbial decomposition for 2 to 3 years in a litter bag (Osono and Takeda 2001b).

Inocula for each assessment were cut out of the margin of the growing colonies on 2% malt extract agar with a sterile cork borer (5.5mm diam) and placed on the center of Petri dishes (9 cm diam) containing 20 ml 2% plain agar. One gram of litter sample, cracked into approx 5 x 5 mm, was sterilized with ethylene oxide gas at 60°C for 3 hours and placed on the surface of the agar. The plates were incubated at 20°C for 16 wks in darkness. After the incubation, leaves were collected, oven-dried at 40°C for 4 days, and weighed. Three plates were prepared for each species. The leaves were then combined and used for chemical analyses. Weight loss of the leaves was determined as a percentage of the original weight. T-

test was used to determine differences between mean values of weight losses of freshly fallen litter and partly decomposed litter.

Chemical analyses

Leaf samples were ground in a laboratory mill (0.5 mm screen) for chemical analyses. The amount of lignin in samples was estimated by gravimetry using hot sulfuric acid digestion (King and Heath 1967). The amount of total carbohydrate was estimated by the phenol-sulfuric acid method (Dubois et al. 1956). The methods are described in Chapter 2.

Lignin/weight loss ratio (L/W) and lignin/carbohydrate loss ratio (L/C) are useful indices of substrate utilization pattern of each fungal species (Osono and Takeda 2002a). L/W and L/C of each fungal species are calculated according to the following equations:

L/W = weight loss of lignin (% of original lignin weight) / weight loss of litter (% of original litter weight)

L/C = weight loss of lignin (% of original lignin weight) / weight loss of carbohydrate (% of original carbohydrate weight)

Experiment 2: Effects of prior decomposition by phyllosphere fungi

Source of fungi and litters

Twelve fungal species were used in this experiment: *Mycena* sp., *M. polygramma* (Basidiomycota), *Xylaria* sp. (anamorph) (xylariaceous Ascomycota), *Alternaria alternata*, *Ascochyta* sp., *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Gliocladium roseum*, *Trichoderma koningii*, *Penicillium citrinum* (Ascomycota other than Xylariaceae), *Mucor hiemalis*, and *Mortierella ramanniana* var. *ramanniana* (Zygomycota). *Xylaria* sp. and *Ascochyta* sp. were phyllosphere species on beech. *A. alternata*, *C. cladosporioides*, *E. nigrum*, *G. roseum* were epiphytic phyllosphere fungi. *Mycena* sp., *M. polygramma*, *T. koningii*, *P. citrinum*, *M. hiemalis*, and *M. ramanniana* were litter fungi (Osono 2002). Details of sources and method of isolation were described in Osono and Takeda (2002a). The detail

of collection and isolation of these fungi are described in Chapter 5. Beech litters used in the decomposition tests were collected by litter-traps in autumn 1997 at the study site.

Decomposition test

An *in vitro* decomposition test was carried out to assess the effect of prior decomposition by two phyllosphere fungi on substrate utilization by twelve fungal species (Osono and Takeda 2002a). *Xylaria* sp. and *Ascochyta* sp. were used as prior decomposers. Three litter types were used in the test: (i) control litter without fungal inoculation (litter C), (ii) litter previously partly decomposed by *Xylaria* sp. (litter X), and (iii) litter previously partly decomposed by *Ascochyta* sp. (litter A).

In the decomposition test of litter C, the disks were exposed to each of 12 fungi for 8 wks without pretreatment by the phyllosphere fungi according to the method described in Chapter 5. Ten plates were prepared for each species. In the decomposition test of litters X and A, the disks were exposed to each of the phyllosphere fungi for 8 wks, then autoclaved and transferred to the plates inoculated with each of 12 fungi and incubated for another 8 wks, according to the same method described above.

Weight loss of the leaf disks was determined as a percentage of the original weight. When analyzing the weight loss of leaf litter, the arcsin transformation was used because the data were in the form of proportions. Analysis of variance (Systat 1992) was used to determine differences among mean values of weight losses of litters C, X, and A. Tukey's honestly significant difference (HSD) test was used for multiple comparisons. T-test was used for *M. polygramma* to determine differences between mean values of weight losses of litters C and X, instead of ANOVA, because no data were obtained from the experiment with *M. polygramma* on litter A due to contamination. Part of the results have already been presented by Osono and Takeda (1999b, 2002a).

Chemical analyses

The amount of lignin in samples was estimated by gravimetry using hot sulfuric acid

digestion (King and Heath 1967). The amount of total carbohydrate was estimated by the phenol-sulfuric acid method (Dubois et al. 1956). The methods are described in Chapter 2.

Lignin/weight loss ratio (L/W) and lignin/carbohydrate loss ratio (L/C) are useful indices of substrate utilization pattern of each fungal species (Osono and Takeda 2002a). L/W and L/C of each fungal species are calculated according to the equations as described above.

Experiment 3: Effect of organic chemical quality and inorganic N addition

The ability of a strain (code GS1-1) of *Xylaria* sp. (anamorph) to decompose leaf litters was assessed by the pure culture decomposition test according to the method described in Chapter 5. Leaf litters of 4 tree species were used, i.e. *Acer mono* Maxim. var. *marmoratum* Hara, *Sorbus alnifolia* (Sieb. et Zucc.) C. Koch, *Quercus mongolica* var. *grosseserrata*, and *Fagus crenata*. In some tests, $(NH_4)_2SO_4$ or $NaNO_3$ was incorporated into the agar (denoted as NH_4 and NO_3 treatment, respectively) at a concentration of 11.76mM N equivalent which is equal to the nitrogen amount in the leaf disks. Weight loss of the leaf disks was determined as a percentage of the original weight. When analyzing the weight loss of leaf litter, the arcsin transformation was used because the data were in the form of proportions. Ten plates were prepared for each test.

The amount of lignin in samples was estimated by gravimetry using hot sulfuric acid digestion (King and Heath 1967). The amount of total carbohydrate was estimated by the phenol-sulfuric acid method (Dubois et al. 1956). The methods are described in Chapter 2.

Relative amount of holocellulose in lignocellulose matrix is a useful index of litter chemical quality. Lignocellulose index (LCI) of litter type is calculated according to the following equation:

LCI = holocellulose conc. / (holocellulose conc. + lignin conc.)

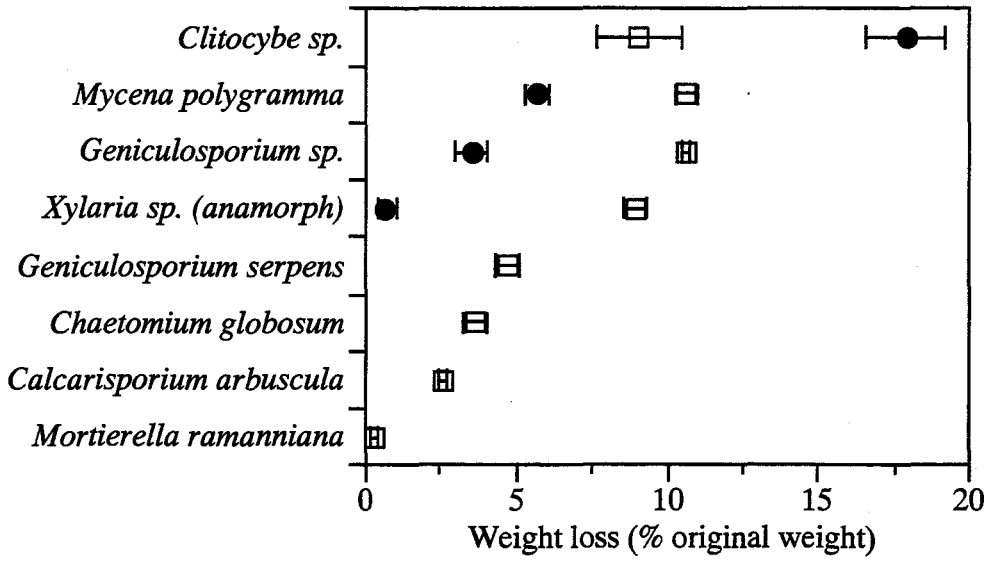

Fig. 6.1 Weight loss (% original weight) of freshly fallen litter (open box) and partly decomposed litter (black circle).

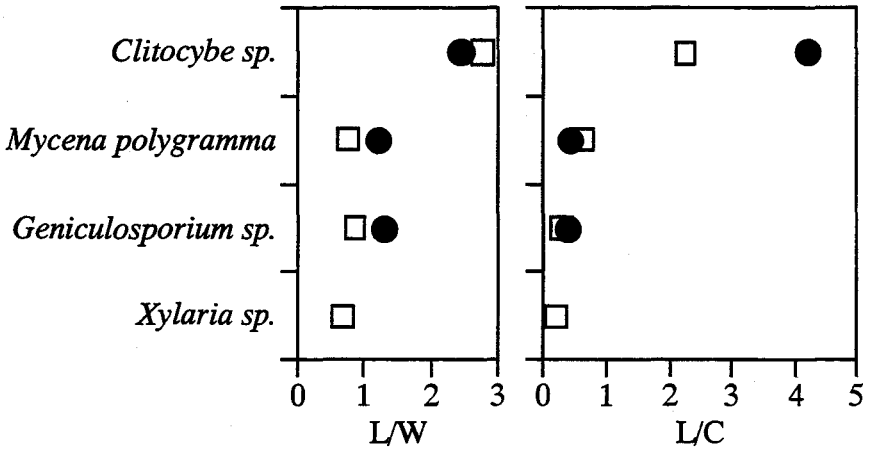

Fig. 6.2 L/W and L/C of fungi inoculated on freshly fallen litter (open box) and partly decomposed litter (black circle).

Results

Decomposition of litter in different decay stages

Freshly fallen litter initially contained 42.8% of lignin and 23.9% of carbohydrate, while partly decomposed litter initially contained 48.2% of lignin and 20.8% of carbohydrate.

Figure 6.1 shows weight loss of freshly fallen litter and partly decomposed litter. *Clitocybe* sp. caused weight loss of partly decomposed litter significantly (p=0.06) higher than

Table 6.1 Weight loss of lignin and carbohydrate, L/W and L/C during decomposition

Fungus	Litter type	Weight loss (% original weight)			
		Lignin	Carbohydrate	L/W	L/C
BASIDIOMYCOTA					
Mycena polygramma	Freshly fallen	8.1	13.2	0.8	0.6
	Partly decomposed	7.0	15.9	1.2	0.4
Clitocybe sp.	Freshly fallen	25.0	11.1	2.8	2.3
	Partly decomposed	43.7	10.4	2.4	4.2
ASCOMYCOTA (XYLARIACEAE)					
Xylaria sp.	Freshly fallen	6.2	30.8	0.7	0.2
	Partly decomposed	5.0	7.2	nd	0.7
Geniculosporium sp.1	Freshly fallen	9.2	32.4	0.9	0.3
	Partly decomposed	4.6	12.3	1.3	0.4

nd L/W of *Xylaria* sp. was not calculated because weight loss of litter was negligible.

that of freshly fallen litter. *Mycena polygramma*, *Geniculosporium* sp., and *Xylaria* sp., on the other hand, caused weight loss of partly decomposed litter significantly ($p<0.05$) lower than that of freshly fallen litter.

Figure 6.2 and Table 6.1 show weight loss of lignin and carbohydrate, L/W, and L/C of 4 species incubated on freshly fallen litter and partly decomposed litter. *Clitocybe* sp. caused marked delignification in partly decomposed litter. L/W and L/C of *M. polygramma* and *Geniculosporium* sp.1 were similar on both litters.

Effects of prior decomposition by phyllosphere fungi

Table 6.2 shows initial chemical composition of litters C, X, and A. Nitrogen concentration was similar among the litters. Concentration of soluble carbohydrate was lower at litters X and A than at litter C. Concentrations of lignin and total carbohydrate were similar between litters C and X, and concentration of lignin was higher and that of total carbohydrate was lower at litter A than at litters C and X. Weight losses (% original weight) of litters X and A for the first 8 wks were 8.2% and 4.7%, respectively.

Figure 6.3 shows weight loss (% original weight) of litters C, X, and A decomposed by the 12 fungal species. Weight loss was higher in the 2 species of the Basidiomycota, *Mycena polygramma* and *Mycena* sp., than in the other 10 species. *Mycena* sp. caused significantly higher weight loss at litter X than at litters C and A. *Xylaria* sp., *Ascochyta* sp., *Gliocladium roseum*, *Penicillium citrinum*, *Epicoccum nigrum*, *Alternaria alternata*, *Cladosporium*

Table 6.2. Concentrations (%) of nitrogen, soluble carbohydrate, lignin, and total carbohydrate in three litter types used in the decomposition test.

Treatment	Code	Nitrogen	Soluble carbohydrate	Lignin	Total carbohydrate
Control, no fungal inoculation	Litter C	1.30	2.9	39.6	34.2
Xylaria sp. inoculation*	Litter X	1.26	1.1	39.2	35.3
Ascochyta sp. inoculation*	Litter A	1.25	0.9	44.9	27.0

* Litters were incubated for 8 weeks at 20°C with each fungus.

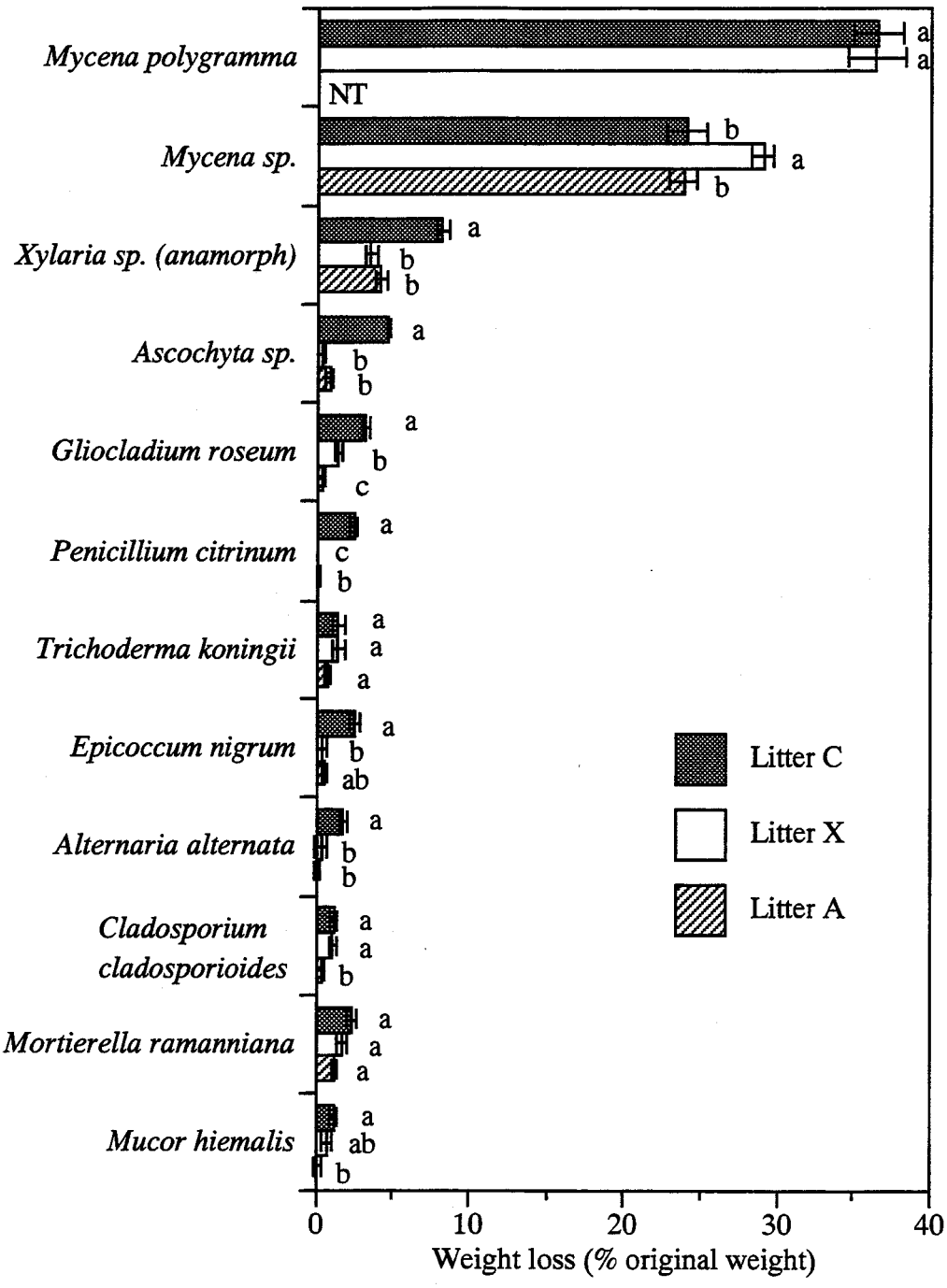

Figure 6.3. Weight loss (% original weight) of litters C, X, and A decomposed by 12 fungal species. For litters C, X, and A, see Table 1. Bars indicate standard errors. The same letters are not significantly different at the 5% level by Tukey's HSD test. NT, not tested.

Table 6.3 Weight loss (% original weight) of leaf litter, lignin, and carbohydrate and L/W and L/C of litters C, X, and A decomposed by *Mycena* sp. and *Mycena polygramma*. For litters C, X, and A, see Table 1.

		Weight loss (% original weight)				
Fungus	Litter type	Litter	Lignin	Total carbohydrate	L/W	L/C
Mycena sp.	Litter C	24.0	27.0	30.2	1.1	0.9
Mycena sp.	Litter X	29.0	48.4	13.2	1.7	3.7
Mycena sp.	Litter A	23.8	34.8	22.1	1.5	1.6
Mycena polygramma	Litter C	36.6	33.2	47.8	0.9	0.7
Mycena polygramma	Litter X	36.5	56.1	23.6	1.5	2.4

cladosporioides, and *Mucor hiemalis* caused significantly lower weight losses at litter X and/or litter A than litter C. *Mycena polygramma*, *Trichoderma koningii*, and *Mortierella ramanniana* caused weight losses that were not significantly different among litters C, X, and A.

Table 6.3 shows weight loss (% original weight) of leaf litter, lignin, and carbohydrate and L/W and L/C of litters C, X, and A decomposed by *Mycena* sp. and *M. polygramma*. *Mycena* sp. caused weight loss of lignin higher and that of total carbohydrate lower at litters X and A than at litter C. L/W and L/C were higher at litters X and A than at litter C. *M. polygramma* caused weight loss of lignin higher and that of total carbohydrate lower at litter X than at litter C. L/W and L/C were higher at litter X than at litter C.

Effect of organic chemical quality and inorganic N addition

Table 6.4 shows weight loss of leaf litter, weight loss of lignin and holocellulose in the litter, and lignin/carbohydrate loss ratio of 4 tree species by the decomposition of *Xylaria* sp. Litter weight loss was highest in *Acer* litter and lowest in *Fagus* litter. Weight losses of lignin and holocellulose were highest in *Acer* litter and lowest in *Quercus* litter. Initial LCI was significantly related to weight loss of lignin ($p<0.05$, r=0.978, n=4) and holocellulose ($p<0.05$, r=0.951, n=4).

Figure 6.4 shows lignin/carbohydrate loss ratio (L/C) of *Xylaria* sp. as a function of initial litter LCI. L/C of *Xylaria* sp. (anamorph) was significantly related to initial litter LCI

Table 6.4 Weight loss (% original weight) of leaf litter and those of lignin and holocellulose in the litter of 4 tree species decomposed by *Xylaria* sp. Values for litter weight loss indicate means ± standard errors (n=10). The same letters show no significant difference at 5% level by Tukey's HSD test.

Litter type	Leaf litter	Lignin	Holocellulose
Acer mono	30.6±0.5a	20.1	45.8
Sorbus alnifolia	22.7±0.8b	11.8	25.9
Quercus mongolica	10.3±1.3c	6.0	3.8
Fagus crenata	8.2±0.4c	10.0	7.1

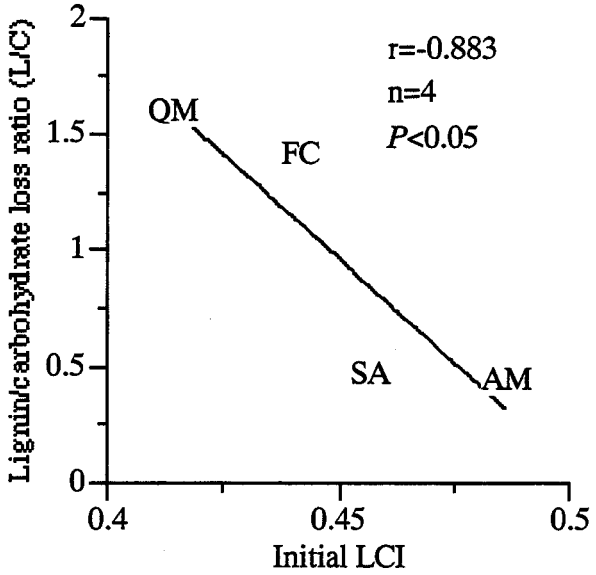

Fig. 6.4 Relationship between initial LCI and lignin/carbohydrate loss ratio (L/C) of *Xylaria* sp. for 4 litter types. AM *Acer mono*, FC *Fagus crenata*, QM *Quercus mongolica*, SA *Sorbus alnifolia*.

(p<0.05, r=-0.967, n=4). The lignin/litter loss ratio (L/W) was not significantly related to initial litter LCI (r=-0.095).

Table 6.5 shows weight loss of beech leaf litter and those of lignin and holocellulose in the litter in NH_4 and NO_3 treatments. In the NH_4 treatment, weight loss of litter was significantly (p<0.05) lower than in the control. Weight loss of lignin was not detected in the NH_4 treatment, while weight loss of holocellulose was higher than in the control. In the NO_3 treatment, weight loss of litter was significantly (p<0.05) higher than in the control. Weight loss of lignin was lower in the NO_3 treatment than in the control, while weight loss of holocellulose was higher than in the NH_4 treatment and in the control.

Table 6.5 Weight loss (% original weight) of leaf litter and those of lignin and holocellulose in the litter of *Fagus crenata* decomposed by *Xylaria* sp. in NH_4 and NO_3 treatments. Values for litter weight loss indicate means ± standard errors (n=10). The same letters show no significant difference at 5% level by Tukey's HSD test. In the Control treatment, the fungus was inoculated to leaf litter without addition of inorganic nitrogen. Weight loss of lignin in NH_4 treatment was not detectable.

Treatment	Leaf litter	Lignin	Holocellulose
NH_4	4.4±0.4c	-	22.4
NO_3	12.9±0.3a	7.8	28.4
Control	8.2±0.4b	10.0	7.1

Discussion

Decomposition of litter in different decay stages

Clitocybe sp. caused higher weight loss of litter and lignin on partly decomposed litter than on freshly fallen litter, indicating that the fungus physiologically adapted for selective delignification in partly decomposed litter. This is consistent with the field observation that *Clitocybe* sp. caused marked bleaching and lignin decomposition on partly decomposed litter materials rather than on freshly fallen litter. Freshly fallen litter contained more carbohydrate and less lignin was energetically suitable substrata for fungi than partly decomposed litter, but *Clitocybe* sp. caused lower weight loss on freshly fallen litter than on partly decomposed litter. This contradiction may be ascribed to the reduction of hyphal growth by suppressive agents such as phenolic compounds (Dix 1979) that are richly contained in freshly fallen leaves (Osono and Takeda 2001b). *M. polygramma*, one of the most frequent species in the study site (Chapter 3), removed lignin and holocellulose simultaneously in freshly fallen litter and partly decomposed litter.

Litter decomposition by two xylariaceous fungi, *Geniculosporium* sp. and *Xylaria* sp., was reduced on partly decomposed litter compared to freshly fallen litter. As the degree of selective delignification and litter decomposing ability of *Xylaria* sp. was dependent on ratio of lignin to total carbohydrate within the substrata (Osono and Takeda 2001a), partly decomposed litter that has higher lignin to carbohydrate ratio was less suitable for the growth of these xylariaceous fungi than freshly fallen litter. The result supports the suggestion of Osono and Takeda (2001b) that litter decomposing activity of these fungi would be lower during the mobilization phase of nitrogen and phosphorus when LCI decreased (i.e., the ratio of lignin to carbohydrate increased).

The weight loss of freshly fallen litter by *M. polygramma* was lower than the previous report (Osono and Takeda 2002a, Chapter 5). The previous and present experiments differed in terms of sterilization method of litter (autoclaving vs gas sterilization), incubation

Table 6.6 Effect of sterilization method on weight loss of litter (% original weight) during *in vitro* decomposition

	Autoclaving	Gas sterilization (ethylene oxide)	Probability
Mycena polygramma	23.1 (0.1)	13.6 (1.2)	$P<0.05$
Xylaria sp.	9.6 (0.8)	6.5 (0.3)	$P=0.10$

Beech litter cut into 1 cm width was inoculated with fungi and incubated at 20°C for 16 wks in darkness. Standard errors in parenthesis (n=3).

period (8 wks vs 16wks), and litter sample preparation (leaf disk with diameter of 20 mm vs fragmented litter of approx 5 x 5 mm). Of these differences, the method of litter sterilization was considered as important, because the autoclaving under high temperature and pressure may cause significant alteration of litter chemistry. The preliminary experiments assessed the effect of sterilization method indicated that decomposition was faster on litter autoclaved than litter sterilized with ethylene oxide gas (Table 6.6). Hering (1967) and De-Boois (1976) also reported that decomposition was enhanced when litter was sterilized with autoclaving rather than gamma irradiation.

Effects of prior decomposition by phyllosphere fungi

The prior decomposition by two phyllosphere fungi retarded litter decomposition or had no significant effect on subsequent decomposition by *Xylaria* sp. and the 9 other species in the Ascomycota and the Zygomycota. The decrease in weight losses in litters X and A compared to litter C is probably ascribable to the prior consumption of non-lignified holocellulose and soluble carbohydrates by *Xylaria* sp. and *Ascochyta* sp., as soluble carbohydrate content in litters X and A was lower than in litter C. Furthermore, the weight losses decreased when *Xylaria* sp. and *Ascochyta* sp. were inoculated to litters previously decomposed by themselves, indicating that litter decomposition by phyllosphere species made the substratum unsuitable for themselves. Therefore, by consuming the readily available resources in litter, phyllosphere fungi may affect the colonization of competitors that have a similar requirement for these resources. This is consistent with the suggestion of Osono and Takeda (2001b) that the competitive interactions between fungal colonizers may be one of the

important factors causing the fungal succession on decomposing litter.

The prior decomposition by two phyllosphere fungi affected the substrate utilization patterns of two *Mycena* species, shifting from simultaneous removal of lignin and carbohydrates (L/W and L/C nearly equal to one) to selective delignification (L/W and L/C more than one). This suggests that some litter inhabiting species in the Basidiomycota may be physiologically adapted to the selective removal of lignin and related recalcitrant substances remaining in litters previously partly decomposed by phyllosphere fungi.

The change of the substrate utilization pattern of *Mycena* spp. on the previously decomposed litters is difficult to explain. A possible explanation is the regulatory effects of carbon and nitrogen nutrition on the degree of selective delignification, which have been reported in some cases. For example, lignin decomposition by wood decay fungi depends on the availability of non-lignified carbohydrates (Kirk et al. 1976); *Xylaria* sp. decomposed lignin more selectively in litter that had a higher lignin to carbohydrate ratio (Osono and Takeda 2001a). This explanation can be applied to litter A in which the lignin to carbohydrate ratio was higher than in litter C, but seems to be inappropriate for litter X that has similar lignin to carbohydrate ratio to litter C.

Effect of organic chemical quality and inorganic N addition

Initial LCI of litter showed a regulatory effect on lignin decomposition by *Xylaria* sp. under the incubation condition. LCI of litter is an index of relative availability of holocellulose in lignocellulose matrix to the fungus. In litters with higher LCI, amount of holocellulose bounded to lignin is relatively low and non-lignified holocellulose is relatively high. As fungal delignification requires a growth substrate such as non-lignified cellulose or glucose (Kirk et al. 1976), *Xylaria* sp. decomposed lignin and holocellulose faster in these litters with higher LCI. On the other hand, in litters with lower LCI, more holocellulose is protected by lignification and available holocellulose necessary for lignin decomposition is relatively low (Reid 1991). As a result, lignin and holocellulose decomposition rate by *Xylaria* sp. was lower in these litters.

Relative utilization pattern of lignin and holocellulose by *Xylaria* sp. (anamorph), expressed as lignin/carbohydrate loss ratio (L/C), was related to litter LCI for 4 litter types. This indicates that the L/C of the fungus was typically changed in relation to the relative availability of lignin and holocellulose in the litter: the fungus decomposed holocellulose in preference to lignin in litters with higher LCI more so than in litters with low LCI. This result is, however, based on a limited number of observations (only one fungus and 4 litter types were used) and further study is needed to assess whether the finding can be applied to other fungal species and other litter types that have wide range of LCI.

Exogenous inorganic nitrogen addition showed a regulatory effect on leaf litter, lignin and holocellulose decomposition by *Xylaria* sp., and the effect of NH_4 and NO_3 was similar on the suppression of lignin decomposition and the stimulation of holocellulose decomposition but its extent was different. There can be two explanations for this difference. Firstly, nitrogen additions caused a biochemical suppression of enzymes associated with lignin decomposition (Fenn et al. 1981) and NO_3 would be less effective than NH_4. Secondly, stimulated holocellulose consumption by nitrogen additions resulted in a rapid exhaustion of available carbohydrates necessary for lignin decomposition (Reid 1991). In NO_3 assimilation, the fungus converts NO_3 to NH_4 for protein production. This conversion requires more energy than direct NH_4 assimilation. Thus the fungus should decompose lignocellulose matrix in addition to non-lignified holocellulose to obtain energy necessary for NO_3 assimilation.

General Discussion and Conclusion

This study demonstrated the ecology and functioning of fungal community on decomposing litter of Japanese beech. Firstly, the methodology of fungal isolation was developed (Chapter 1). Then, the mycological survey was carried out for microfungi on individual leaf (Chapter 2) and for macrofungi on forest floor (Chapter 3). Field evidences were obtained from the bleached leaves and litters that indicated these fungi participated in litter decomposition (Chapters 2 and 3). These results provided the basis to evaluate the relationship between fungal succession and chemical changes during decomposition (Chapter 4) and the functional biodiversity of fungi (Chapters 5 and 6). In this chapter, I discuss the mechanism of fungal decomposition of leaf litter and the implication for soil humus accumulation.

Methodology of decomposer fungal community

Ecologically, litter-inhabiting fungi are divided into two groups, component-restricted fungi and component-non-restricted fungi (Cooke and Rayner 1984). Component-restricted fungi are limited in extent by the physical boundaries of the substrata they occupy. On the other hand, component-non-restricted fungi often have large mycelia that are unrestricted by the spatial limitations of individual substrata. For component-non-restricted fungi, it is the entire litter system, not its individual components, which provides a habitat, and their proper study requires sampling methods to be scaled up accordingly. Thus, different methods are needed for each of these groups to investigate the role of fungal community in litter decomposition, as both component-restricted and component-non-restricted fungi take part in decomposition. Hering (1965, 1966, 1972) and Saito (1956, 1957, 1966) successfully applied this approach for the study of fungal decomposition on leaf litter. The present study also confirmed the importance and usefulness of applying different methods to each of component-restricted and component-non-restricted fungi to follow the mechanism of fungal decomposition of leaf litter.

Table D.1 Relation of fungal succession with characters of decomposition phases of beech leaf litter: a summary.

Decomposition phase	Leaching phase	Immobilization phase	Mobilization phase
Time (month)	0-5	5-21	21-35
Nitrogen dynamics	Leaching	Immobilization	Mobilization
C/N		55 → 26	26 → 24
L/N		45 → 27	27 → 26
Lignocellulose index (LCI)		0.45 → 0.34	0.34 → 0.33
Function of fungi	Rapid exhaustion of soluble carbohydrates	Selective holocellulose decomposition	Simultaneous decomposition of holocellulose and lignin
Fungal ingrowth		High	Low
Fungal succession			
- Functional group	Xylariaceous endophytes, Epiphytes or primary saprophytes (Ascomycota)	Xylariaceous endophytes (Ascomycota)	Litter-decomposing Basidiomycota Xylariaceous endophytes (Ascomycota)
- Associated group		Litter inhabitants (Ascomycota, Zygomycota)	Litter inhabitants, Secondary sugar fungi (Ascomycota, Zygomycota)

Litter decomposition and fungal community dynamics

Table D.1 shows relationship between fungal succession and organic chemical changes and nitrogen dynamics in beech litter during 3 year decomposition. Decomposition of freshly fallen leaves was carried out by epiphytic phyllosphere fungi that colonized on not only the surface but the interior of freshly fallen leaves. Hudson (1968) called these epiphytic fungi as 'primary saprophytes' because they depend their growth on soluble carbohydrate richly contained in freshly fallen leaves. Soluble carbohydrates were quickly utilized by epiphytes and exhausted in the litter.

The loss of soluble carbohydrate caused by not only fungal metabolism but leaching. The contribution of microbial activity and leaching to litter weight loss was unclear in beech leaf litter. There has been only one such estimation on oak litter that lost 9.3% of its initial dry weight during the first 6 weeks (Tietema and Wessel 1994). About 64% of this weight loss was attributed to microbial respiration and 21% to leaching of dissolved organic compounds.

Epiphytes (or primary saprophytes) disappeared as the exhaustion of soluble carbohydrate. Instead, xylariaceous endophytes such as *Xylaria* sp. and *Geniculosporium* sp. increased their abundance and frequency on decomposing litter because of their ability to utilize residual structural components such as lignin and holocellulose. Therefore, the survival of epiphytes and endophytes on beech leaf litter was associated with their energy requirement.

Organic chemical changes during decomposition were related to ingrowth and succession of xylariaceous fungi and basidiomycetous fungi. These fungi differed in their substrate utilization efficiency. The Xylariaceae had the priority due to not only their endophytic colonization of living or senescent leaves but also their effective utilization of holocellulose over lignin. The xylariaceous Ascomycota attacked holocellulose preferentially over lignin and increased their biomass. However, this resulted in the increase of lignin and related substances in litter making the resource quality unsuitable for themselves. The low litter LCI (i.e., relative amount of holocellulose over lignin plus holocellulose) resulted in the increase in their lignocellulose utilization efficiency and in the decrease of decay rate of lignin and holocellulose.

Conversely, the decreased litter LCI was suitable for the Basidiomycota because of high lignocellulose utilization efficiency. Litter-decomposing Basidiomycota had the extensive ability to decompose lignin and secondary lignin-like substances synthesized during decomposition. These fungi attacked lignin and holocellulose simultaneously or removed lignin selectively. These fungi gradually increased their abundance as the decrease of LCI and replaced the Xylariaceae at later stage of litter decomposition.

Another pattern of fungal succession from primary saprophytes to litter inhabitants and soil fungi was observed on litter surface. This succession had little effects on the litter decomposition, because these fungi had limited ability to attack lignin and lignified holocellulose and depended for their growth on non-lignified holocellulose or soluble carbohydrate. Therefore, the succession of these fungi was difficult to relate to decomposition of lignin and holocellulose in litter. The majority of mycological study has focused on this fungal succession, making it difficult to relate the functioning of fungal community to litter decomposition.

Nitrogen dynamics

Nitrogen (N) dynamics was regulated by fungal decomposition of lignin and holocellulose during 3-year decomposition of beech leaf litter. Nitrogen dynamics showed two phases, immobilization and mobilization phases during decomposition. In a typical case, nitrogen shows leaching, immobilization, and mobilization phases (Staaf and Berg 1982). In this study, the litter bags set in December were under snow cover over a 5 months. The leaching process occurred on freshly fallen leaves during the winter period or at the snow melting in Spring (Takeda et al. 1987). Epiphytes were not crucial for the N leaching in spite of their frequent occurrence on the freshly fallen leaves because the leaching is a physical process.

Nitrogen immobilized in decomposing litter from 6th to 21st months as the growth of xylariaceous fungi. Immobilization of N occurred until lignin to nitrogen ratio (L/N) reached at about 25. The chemical background of N immobilization in litter is still unclear. Nitrogen

bounded in fungal biomass as chitin and proteins accounted for only 1.5% to 1.8% of total litter N. These values were too small to explain the N immobilization in litter. On the other hand, it has been assumed that N is incorporated into the recalcitrant secondary substances (Berg 1986). The result of present study suggested holocellulose hydrolysis and partial lignin modification by xylariaceous fungi caused the binding of N to lignin to produce a presumable lignin-N complex. The critical value of L/N at which N mineralization occurred supported the view that the formation of lignin-N complex is the major process of N immobilization. Osono and Takeda (unpublished) found that critical values of L/N of 14 tree species converged to about 25 during 3 year decomposition in the study site, irrespective of their initial values.

Furthermore, the presence of lignin-N complex was supported by the finding that decomposition of lignin and related humic substances by the Basidiomycota was associated with N mineralization. The bleached litter produced by *Clitocybe* sp. was a typical case demonstrating the correspondence between highly selective delignification and rapid N mineralization.

Fungal decomposition and soil humus accumulation

The selective decomposition of holocellulose by the Ascomycota resulted in the accumulation of recalcitrant lignin-N complex in forest soils, whereas the delignification by the Basidiomycota resulted in the mineralization of carbon and nitrogen bounded in the recalcitrant forms. A similar result was obtained in a pure culture decomposition test using *Larix* needle litter (Osono et al. submitted). Therefore, it is suggested the relative importance of the Ascomycota and the Basidiomycota in decomposer fungal community may determine the type of organic matter accumulation in forest soils.

However, decomposition rates, organic chemical and nutrient dynamics of beech litter, and pattern of fungal succession were similar over 3-year period between moder and mull soils. This result suggested the initial phase of litter decomposition up to 3 year period had negligible effect on the development of moder and mull soils. Instead, a possible relationship between bleaching of partly decomposed litter by the Basidiomycota and mull

soil formation is suggested in Chapter 3. This suggestion is supported by the finding of Gourbière (1982) that white rot fungi caused litter weight loss when the decay rate slowed down and the litter reached at Fb layer at the 9th year after litter fall. Previous works that suggested the importance of soil animals on soil humus accumulation (Bal 1982), and the present study proposed a possible importance of fungi in the soil humus development.

Conclusion

The decomposition of lignin and holocellulose and immobilization and mobilization of nitrogen were driven by the successive colonization and substrate utilization of the xylariaceous Ascomycota and the Basidiomycota that had different substrate utilization efficiencies. A possible importance of fungi in the soil humus development was proposed, but more studies are needed to relate the fungal decomposition of leaf litter to the accumulation of soil organic matters in forest soils.

Summary

Litter decomposition is an important process for the maintenance of functioning and biodiversity in forest ecosystems. Chemical aspects of the decomposition processes have been studied intensively while the role of decomposer fungi has been paid little attention. There has been no study that relates the ecology and functioning of fungal community to the organic chemical and nutrient dynamics during litter decomposition. The purpose is to clarify the mechanism of fungal decomposition of leaf litter of Japanese beech (*Fagus crenata*). The study was carried out in a cool temperate forest in Kyoto, Japan. Firstly, methodology of fungal isolation was developed (Chapter 1). Then, mycological survey was carried out for microfungi on individual leaf (Chapter 2) and for macrofungi on forest floor (Chapter 3). Based on these results, the relationship between fungal succession and chemical changes during 3 year decomposition was investigated (Chapter 4). Litter decomposition potentialities of the fungi were verified with pure culture decomposition tests (Chapters 5 and 6). These results indicated the decomposition of lignin and holocellulose and immobilization and mobilization of nitrogen in litter were driven by the successive colonization and substrate utilization of the Xylariaceae and the Basidiomycota that had different substrate utilization efficiencies.

Chapter 1

A methodological survey was carried out on the effects of nutrient media and incubation period on number of species and species composition of fungi on beech leaf litter. Leaf litter was incubated on nutrient poor (LCA) and nutrient rich (PDA) media. Fungi isolated were tested their growth rates on LCA and PDA. Significantly larger numbers of species were obtained on LCA than on PDA. On PDA, fast-growing fungi were isolated selectively. Ninety percent of total species were isolated during 3-week incubation. Therefore, the incubation on the nutrient poor medium (LCA) for two months was successful for the description of mycobiota on beech leaf litter.

Chapter 2

Phyllosphere fungi occur on various litters but the ecology of these fungi on leaf litter has received little attention. To investigate the occurrence, colonization, and succession of phyllosphere fungi and their bleaching activity on beech leaf litter, fungi were isolated from living, senescent, freshly fallen, and decomposing leaves and from bleached leaves by surface sterilization and washing methods. A total of 18 and 47 fungal species were isolated from the interior and surface of living and senescent leaves, respectively, and fifteen frequent species were regarded as phyllosphere fungi. These phyllosphere fungi were divided into three groups according to their frequency on freshly fallen and decomposing leaves. Nine species (Group I) occurred frequently on decomposing leaves, two species (Group II) on freshly fallen leaves only, and four species (Group III) were frequent on living or senescent leaves only. Colonization of sterilized freshly fallen leaves by phyllosphere fungi was investigated to test their ability to infect litter directly after litter fall. Frequencies of four species were lower on sterilized leaves than on unsterilized leaves, whereas frequencies of other species did not differ between sterilized and unsterilized leaves. Successional trends of endophytes and epiphytes were observed during decomposition from freshly fallen to decomposing leaves. The sum of frequencies of endophytes decreased temporarily on freshly fallen leaves and increased on decomposing leaves. The sum of frequencies of epiphytes decreased from freshly fallen to decomposing leaves.

Mycobiota and chemical composition of bleached and non-bleached portions were studied on decomposing litter. By surface sterilization method, two phyllosphere species in the Xylariaceae *Xylaria* sp. (anamorph) and *Geniculosporium* sp.1 were frequently isolated in both portions. Frequency of *Xylaria* sp. (anamorph) was significantly higher in the bleached portion than in the non-bleached portion. In the bleached portion, lignin concentration was lower than in the non-bleached portion, indicating that *Xylaria* sp. (anamorph) and *Geniculosporium* sp.1 took part in lignocellulose decomposition.

Chapter 3

Species composition and mycelial abundance of the Basidiomycota were investigated on upper (moder) and lower (mull) sites of a forest slope with reference to bleaching activity. The bleaching of leaf litter is analogous to white rot of wood that is due to removal of lignin as well as cellulose. Frequency of fruit body and number of species of litter decomposing fungi were higher in lower site than in upper site. The most frequent species were *Mycena filopes* and *M. polygramma* in both sites. Eighty percent of fruit body collected emerged from the L layer. Phenology of fruit body had two peaks: the first peak during late-May to late-June and the second peak during mid-September to early November. Total and clamp-bearing fungal biomass was highest at L layer in both sites. Clamp-bearing biomass in upper site increased rapidly on November. The bleached litter was mostly encountered on L layer and associated with fruit body of *Clitocybe* sp. Hyphal length in the bleached litter was about 5 time higher than in surrounding non-bleached litter. Concentration of lignin was lower and those of nutrients (N, P, K, Ca, Mg) were higher at the bleached litter than at the non-bleached litter. Inorganic nitrogen pool sizes and net N mineralization rate were higher at the bleached litter than at the non-bleached litter. Although a variety of fungi were isolated from the bleached litter by washing method, *Clitocybe* sp. produced the dominant effects as this fungus brought about similar changes when they decomposed litter in pure culture. A possible relationship between the bleaching activity of the Basidiomycota and soil humus accumulation was suggested.

Chapter 4

Decomposition processes of beech leaf litter were studied with the litter bag method over a 3 year period. Organic chemical and nutrient dynamics and fungal ingrowth and succession were followed on upper (moder) and lower (mull) sites of a forest slope. Litter decomposition rates were similar between the sites. Nutrients were categorized into 2 types according to their dynamics in the decomposing litter: nitrogen and phosphorus showed two phases, the immobilization (0-21 month) and the mobilization phases (21-35 month), while

potassium, calcium, and magnesium showed only the mobilization phase. The rate of loss of organic chemical constituents was lignin < holocellulose < soluble carbohydrate < polyphenol in order. Changes in lignocellulose index (LCI), the ratio of holocellulose in lignin and holocellulose, were significantly correlated to changes in concentrations of nitrogen and phosphorus during decomposition. During the immobilization phase, increase in total fungal biomass contributed to the immobilization of nitrogen and phosphorus. The proportion of clamp-bearing fungal biomass (biomass of the Basidiomycota) to total fungal biomass increased as the decomposition proceeded and was significantly correlated with LCI. Two species in the xylariaceous Ascomycota were frequently isolated by surface sterilization method from decomposing litter collected at the 11th month. The organic chemical, nitrogen, and phosphorus dynamics during decomposition were related to the ingrowth, substrate utilization, and succession of the Xylariaceae and the Basidiomycota. Twenty-one species in the other Ascomycota and the Zygomycota isolated by washing method were classified into three groups based on their occurrence patterns: primary saprophytes, litter inhabitants, and secondary sugar fungi. These species showed different responses to LCI and soluble carbohydrate concentration of litter between the groups.

Chapter 5

The litter decomposing ability of 79 fungal isolates (41 genera, 60 species) was assessed with pure culture decomposition test. The isolates were collected qualitatively during a 21-month period. Loss of original weight of sterilized litter ranged from 0.1% to 57.6%. Six isolates in the Basidiomycota caused high weight losses ranging from 15.1% to 57.6%. Fourteen isolates in *Xylaria* and *Geniculosporium* (the Xylariaceae and its anamorph) also caused high weight losses ranging from 4.0% to 14.4%. Other isolates in the Ascomycota and associated anamorphs and in the Zygomycota caused low weight losses on mean. Six fungi in the Basidiomycota, and all in the Xylariaceae showed a bleaching activity of litter and caused lignin and carbohydrate decomposition. Mean lignin/weight loss ratios (L/W) and lignin/carbohydrate loss ratios (L/C), were 0.9 and 0.7 for the Basidiomycota and 0.7 and 0.4

for the Xylariaceae, respectively. Significant differences were found in L/W and L/C between the two groups when the result of *Xylaria* sp. that showed marked delignification was excluded. These differences in lignin and carbohydrate utilization patterns were discussed in relation to the structural and the chemical properties of the decomposed litter and to the implication to organic chemical changes during litter decomposition.

Nutrient contents (N, P, K, Ca, Mg) of leaf litter decomposed by selected 19 fungal isolates (6 of the Basidiomycota, 7 of the xylariaceous Ascomycota and 6 of the other Ascomycota) were compared among the taxonomical groups. The mean N concentration in litter decomposed by Basidiomycota fungi (litter B) was not significantly different from that decomposed by the xylariaceous Ascomycota (litter XA) but was significantly higher than that decomposed by the other Ascomycota (litter OA). The mean P concentration of litter XA was significantly higher than that of litters B and OA. The mean Mg concentration of litter B was significantly lower than that of litters XA and OA. No significant differences were found in the mean K and Ca concentrations among the litters. These results were discussed in relation to the role of these fungi in nutrient dynamics during litter decomposition.

Chapter 6

The ability of 4 species to decompose freshly fallen litter and partly decomposed litter of beech was assessed. The partly decomposed litter was exposed to natural microbial decomposition for 2 to 3 years in litter bags. *Clitocybe* sp. caused weight loss of partly decomposed litter higher than that of freshly fallen litter. *Mycena polygramma*, *Geniculosporium* sp., and *Xylaria* sp. caused weight loss of partly decomposed litter lower than that of freshly fallen litter. *Clitocybe* sp., *Geniculosporium* sp., and *Xylaria* sp. caused more selective delignification on partly decomposed litter than on freshly fallen litter. *M. polygramma* removed lignin and holocellulose simultaneously on both litters.

Changes in litter quality due to pretreatment of leaf litter by phyllosphere fungi may affect its subsequent decomposition by succeeding fungi. The purpose is to clarify the effects of prior decomposition of leaf litter by two phyllosphere fungi of beech, *Xylaria* sp. and

Ascochyta sp., on substrate utilization of 12 fungal species in the Basidiomycota, the Ascomycota, and the Zygomycota, was investigated in a laboratory experiment. *Mycena* sp. caused significantly higher weight loss in litter previously partly decomposed by *Xylaria* sp. than in control litter without fungal inoculation and litter previously partly decomposed by *Ascochyta* sp., while prior decomposition retarded litter decomposition or had no significant effect on 11 other species. Prior decomposition by phyllosphere fungi affected the substrate utilization patterns of two *Mycena* species in the Basidiomycota, shifting from simultaneous removal of lignin and carbohydrates to selective delignification.

The effects of organic chemical quality of litters and exogenous inorganic nitrogen (NH_4^+ and NO_3^-) addition were investigated on the ability of *Xylaria* sp. to decompose lignin and carbohydrate *in vitro*. Lignocellulose index (LCI) was significantly related to weight loss of lignin and lignin/carbohydrate loss ratio (L/C) for 4 litter types tested. In NH_4 and NO_3 addition treatments, lignin decomposition was completely and partially suppressed, respectively.

Synopsis in Japanese

落葉の分解過程は、一次生産者である森林植物への養分物質の供給、および養分・水分の貯蔵庫となる土壌有機物の形成を通して、森林の生態系機能と、生態系内における生物多様性の維持に深く関わっている。これまでの研究から、落葉分解の化学的な側面とも、すなわち分解者生物にとってのエネルギー源となるリグニンやセルロースなど有機物の組成変化や、窒素やリンといった養分物質の動態について成果が得られてきた。一方で、落葉の分解に関わる分解者生物の役割、特に菌類の役割については、その重要性が広く認識されているにも関わらず、これまでほとんど注意が払われてこなかった。

そこで本研究では、分解に関わる菌類の生態や分解機能を明らかにし、それを落葉分解にともなう有機物や養分物質の動態と関連づけることが、森林生態学の中心的課題であると位置づけた。本研究の目的は、菌類による落葉の分解メカニズムを生態学的な観点から明らかにすることである。調査は、京都府北東部の京都大学農学部附属芦生演習林内の冷温帯天然林において、優占樹種であるブナの落葉を材料として実施した。

菌類は微小な生物群であり直接的な観察や計数が困難である。よってまず最初にブナ落葉上の菌類を分離・培養する方法について方法論的な検討を行った（第１章)。

菌類は種組成の記載といった基礎情報に乏しい分野である。そこで、個々のブナ葉上に定着している微小菌類（主に子嚢菌類、接合菌類）の種組成、および林床において複数の落葉に定着している大型菌類（大型の子実体を作る担子菌類）の種組成を明らかにした（第２章、第３章)。また落葉や有機物層が白色化する漂白現象に着目し、落葉の漂白が子嚢菌類や担子菌類によるリグニンの分解の結果として生じることを突き止めた（第２章、第３章)。

これらの成果に基づいて、ブナ落葉の分解実験を調査地において３年間にわたり実施し、分解にともなう菌類の菌糸成長の消長・菌類遷移と、有機物組成（リグニン、ホロセルロース、可溶性糖類、ポリフェノール)、養分物質（窒素、リン、カリウム、カルシウム、マグネシウム）の変化との関連性を明らかにした（第４章)。

これら野外実験で明らかになった菌類による落葉分解パターンを検証するため、実験室内において、滅菌したブナ落葉に分離菌株を接種して分解させる実験をおこなった。多様な種の落葉分解機能を明らかにし（第５章)、さらに分解にともなう落葉の化学的性質の変化が菌類の分解機能に与える影響を評価した（第６章)。

以上の結果をもとに総合考察をおこない、ブナ落葉の分解にともなう有機物と養分物質の動態を、異なる資源利用効率を持つ菌類群の遷移から説明した。異なる資源利用効率を有する菌類群の定着が、落葉分解にともなう養分物質の動態や土壌有機物の形成を決定する主要因であると結論した。

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